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# Decipher the origins of slow wave sleep and paradoxical sleep using a multimodal and integrative approach of the squamates order

Baptiste Barrillot

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**Déchiffrer les origines du sommeil lent  
et du sommeil paradoxal par une  
approche multimodale et intégrée de  
l'ordre des squamates**

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Devant le jury composé de :

Pr Anne Didier, Professeure, Université Claude Bernard, Centre de Recherche en Neurosciences de Lyon, Présidente

Pr Gilles Laurent, Professeur, Max Plank Institute for Brain Research,  
Rapporteur

Dr Markus Schmidt, Praticien Hospitalier et Directeur de Recherche, Schlaf-Wach-Epilepsie-Zentrum,  
Rapporteur

Dr Gabrielle Girardeau, Chargée de Recherche Inserm, Institut du Fer à Moulin,  
Examinatrice

Dr Anthony Herrel, Directeur de Recherche CNRS, Museum National d'Histoire Naturelle,  
Examineur

M. Paul-Antoine Libourel, Ingénieur de Recherche CNRS, Centre de Recherche en Neurosciences de Lyon, Directeur de thèse



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# Déchiffrer les origines du sommeil lent et du sommeil paradoxal par une approche multimodale et intégrée de l'ordre des squamates.

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## Résumé

Le sommeil est présent dans l'ensemble du monde animal. Il a été identifié chez toutes les espèces étudiées à ce jour. Ce phénomène complexe et mystérieux a été divisé en deux états dans les années 50 chez l'humain et le chat, le sommeil lent, défini par une activité cérébrale caractérisée par des ondes lentes de fortes amplitudes et une diminution globale des autres paramètres physiologiques par rapport à l'éveil, et le sommeil paradoxal défini lui par une activité cérébrale similaire à celle de l'éveil dans un corps endormi et associé notamment à des saccades oculaires (ce qui lui a donné son nom anglais, rapid eye movement sleep) et une profonde atonie musculaire. En se basant sur le phénotype physiologique unique de ces états, cette dualité a ensuite été identifiée chez tous les mammifères terrestres et les oiseaux étudiés. Cependant, leur origine évolutive reste incertaine. Est-ce que ces deux états de sommeil résultent d'une évolution convergente, qui impliquerait une apparition indépendante des deux états chez les oiseaux et les mammifères, ou d'un phénotype ancestrale qui existerait déjà chez l'ancêtre commun des amniotes ? Longtemps envisagée comme étant uniquement présente chez les homéothermes, et donc issu d'une évolution convergente, de récents travaux chez le lézard ont remis en question cette ancienne hypothèse en montrant l'existence de deux états électrophysiologiques distincts pendant le sommeil chez deux espèces de lézards (*Salvator merianae* & *Pogona vitticeps*). L'ordre des squamates (les lézards et serpents), et plus largement les reptiles non-aviens, sont à une position clé d'un point de vue évolutif pour retracer l'origine des deux états de sommeil puisqu'il partage le même ancêtre commun que les mammifères et les oiseaux. De plus, même si les phénotypes des deux espèces de lézards étudiées présentent certaines similitudes comme la présence de pointes ondes caractéristiques de l'un des états de sommeil, les deux états de sommeil identifiés chez les squamates ont révélé de fortes distinctions entre les deux espèces étudiées, mais plus important encore, avec le phénotype classique des états de sommeil mammalien. Dans ces travaux de thèse, nous discutons tout d'abord de l'importance ne pas chercher à calquer des caractéristiques du sommeil des mammifères chez des espèces qui ne sont pas nécessairement capable de l'exprimer. Dans un second temps et dans le but d'étendre notre compréhension de l'origine et l'évolution de ces deux états de sommeil, nous avons enregistré et caractérisé le sommeil de trois espèces différentes de squamates. Dans une première étude, nous apportons de nouvelles données sur le phénotype de sommeil du *Pogona vitticeps*, en mesurant l'évolution du tonus musculaire, de la fréquence cardiaque, l'ouverture des yeux, les mouvements du sommeil, couplés à l'enregistrement des potentiels de champs locaux de la crête ventriculaire dorsale. Cette espèce montre une augmentation du nombre de mouvements oculaires pendant le second état de sommeil, mais nous n'avons pas révélé de différences significatives des autres paramètres physiologiques mesurés entre les deux états de sommeil de cette espèce. De plus, nous avons décrit pour la première fois un comportement de fermeture unilatérale des yeux pendant l'éveil associée à une asymétrie de l'activité cérébrale avec l'hémisphère cérébral controlatéral de l'œil fermé exprimant les mêmes pointes ondes que celles mesurées pendant le premier état de sommeil alors que l'autre hémisphère conserve une activité cérébrale caractéristique d'éveil. Cette découverte ouvre de nouvelles questions sur la nature même des ondes lentes chez les squamates ou sur la potentielle existence d'un sommeil unilatéral chez ces derniers. Nous avons également caractérisé un autre *Agamidae*, une espèce proche du *Pogona vitticeps*, l'agame étoilé, *Stellagama stellio*. Chez cette espèce, nous avons trouvé la même alternance de deux états de sommeil cérébraux et la même rythmicité associée identifiée chez le *Pogona vitticeps*, suggérant une origine ancestrale dans le

groupe des *Agamidae*. Le phénotype des états de sommeil identifiés chez cette espèce est également très similaire à ceux du *Pogona vitticeps* avec un premier état défini par des pointes ondes caractéristiques et un second état marqué lui par une activité cérébrale similaire à ce qui peut être enregistré pendant l'éveil. Cependant, comme le *Pogona vitticeps*, cette espèce n'a pas montré de changement physiologiques hormis une augmentation des mouvements oculaires pendant le second état de sommeil. Enfin, nous nous sommes intéressés à un autre groupe de squamates jamais enregistré, les *Scincimorpha*, via le *Broadleysaurus major*. Etonnamment, bien que cette espèce montre des signes comportementaux de sommeil, nous n'avons pas pu démontrer l'existence de deux états cérébraux distincts pendant leur sommeil. Cependant, elle présente une lente oscillation de l'activité cérébrale qui rappelle la rythmicité dans l'alternance des états cérébraux du sommeil des *Agamidae*. Bien que ces travaux présentent certaines limites, en particulier relatif au nombre d'animaux étudiés, ils questionnent l'homologie entre les deux états de sommeil trouvés chez les *Agamidae* et le sommeil paradoxal et sommeil lent des mammifères. En effet, bien que deux états cérébraux puissent co-exister pendant le sommeil chez les *Agamidae* et les *Teiidae*, il semblerait que ce phénotype ne soit pas exprimé par tout l'ordre des squamates. De plus, les changements physiologiques associés au sommeil des mammifères semblent être moindres voire inexistants chez les squamates. L'atonie musculaire caractéristique du sommeil paradoxal chez les homéothermes par exemple ne semblent pas exister chez les squamates, de même que les changements de la température cérébrale ou de la fréquence cardiaque. Ces différences suggèrent que la ou les fonction-s du sommeil, ainsi que la nature de ses états cérébraux des squamates pourrai-en-t être différent-e-s de celle-s des mammifères. Cependant, la rythmicité cérébrale retrouvée chez toutes les espèces étudiées au cours de ces travaux suggère qu'il pourrait exister un phénotype de sommeil commun chez les squamates. Existe-t-il chez tous les reptiles non-aviens ? Est-ce que cette oscillation pourrait être une réminiscence de la forme ancestrale des deux états de sommeils des homéothermes ? Ces questions ne pourront être résolues qu'avec une meilleure compréhension des phénotypes de sommeil des reptiles et plus largement du reste du monde animal.

# Decipher the origins of slow wave sleep and paradoxical sleep using a multimodal and integrative approach of the squamates order.

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## Abstract

Sleep is ubiquitous across the animal kingdom. It has been identified in every animal studied to this day. This complex and mysterious phenomenon has been divided in the 1950's into two states – Rapid Eye Movement Sleep and Slow Wave Sleep – in humans and cats. Based on the unique physiological phenotype of those states, this duality has been identified in every land mammal and bird. However, their evolutionary origin remains unclear. For a long time thought to be only present in homeotherms, recent works on lizards challenged this old vision as two distinct electrophysiological sleep states were found into two different species of lizards (*Salvator merianae* & *Pogona vitticeps*). The squamates order (lizards and snakes), and more largely the non-avian reptiles, are at a key evolutionary position to trace the two sleep states origin, as they share a common ancestor with mammals and birds. Besides, the two brain states found in squamates during sleep revealed a strong difference between the two species, but more importantly, with the classical mammalian sleep states phenotype. In this work, we first discuss the importance to stop looking for mammalian features of sleep states in species that are not necessarily able to express it. Moreover, in an attempt to further our understanding of the origin of the two sleep states and how sleep is expressed in squamates, we recorded and characterised sleep in three different species of squamates. In a first study, we provide a larger picture of the sleep phenotype of *Pogona Vitticeps*, by measuring muscle tone, heart rate, eye closure, sleep body movements, in addition to telencephalic local field potential recordings. Notably, except a slight increase in the eye movement density in one state, we reported no clear change of the other physiological variables across the two sleep states. In addition, we described for the first time a unilateral eye closure behaviour related to an asymmetric brain activity. This discovery opens the question on the nature of lizard cerebral slow waves or the potentiality for unilateral sleep to exist in squamates. We also characterised sleep in another *Agamidae*, a sister species of the *Pogona vitticeps*, the starred agama, *Stellagama stellio*. In this species, we found the same sleep state alternance and unique cyclicity reported for *Pogona vitticeps*, suggesting an ancestral pattern through the *Agamidae* group. Finally, we looked at another group of squamates never recorded for sleep, the *Scincomorpha*: *Broadleysaurus major*. Surprisingly, while this species showed sleeping behaviour, we did not manage to demonstrate the existence of two clear cerebral sleep states. However, they present an infraslow oscillation that recalls the *Agamidae* rhythm. Although this work has some limitations, in particular regarding the number of animals studied, it questions the homologies between the two cerebral states found during sleep in the *Agamidae* species and the mammalian rapid eye movement and slow wave sleep. Indeed, although two cerebral sleep states could exist in *Agamidae* and *Teiidae*, it seems not to be similarly expressed across the squamate order. In addition, physiological changes associated with mammalian sleep states tend to be less important or inexistent in lizards, suggesting that the functions and the nature itself of the non-avian reptile cerebral sleep states could differ with the mammalian ones.



## Introduction

### Sleep history and definition

Sleep is universal. It has been a subject of interest for centuries and even ancient Greece philosophers wrote about it (Aristotle, s. d.). So far, every studied species displays a sleeping behaviour. Sleep appears as a quiescent state occurring on a circadian rhythm. But how is this vigilant state defined and what are its functions?

The first component of sleep is its behaviour. The premises of this definition were set in 1912 and were described as follows (Piéron, 1912). Sleep is a vigilant state marked by 1) a prolonged immobility that occurs in 2) a stereotypic location in a stereotypic position that are both species dependant. During this state, the individual has 3) an increase of its arousal threshold meaning that sleeping animals will take more time to react to any type of sensory stimulus compared to wakefulness. Sleep is also 4) a reversible state, this set it apart from other quiescent states (e.g., anaesthesia, torpor, coma, hibernation & brumation). Later this behavioural definition was extended to two other points (Tobler, 1995). Sleep occurs on 5) a circadian rhythm and is 6) homeostatically regulated. This regulation means that when deprived of sleep an individual will go out of its ways to increase its sleep quantity and quality. Based on these points, scientists were able to describe sleep in every studied species so far, from the humble jellyfish to more “complex” organisms (Campbell & Tobler, 1984; Nath et al., 2017; Siegel, 2008).

In the early 1930's, the first reports of electrophysiological recordings of the brain activity during sleep emerge (Gibbs et al., 1935; Loomis et al., 1935, 1937). They described a state of slow, high amplitude waves occurring during the sleep period. They were the first recordings of the typical oscillations of Slow Wave Sleep (SWS). It was later uncovered that those waves originate in mammals from the thalamocortical regions (Steriade et al., 1991). This activity in turn drives a global cortical oscillation synchronising the whole cortex into this sleep's characteristic high amplitude slow waves. The physiology associated with this vigilant state is usually a slowed down version of wake. Respiratory and cardiac frequencies are diminished and regular, eye movements almost never occur, muscle tone is minimal, and body and brain temperature slowly decrease.

It was not until the 1950's, that reports of a second sleep state appeared. This state was first identified based on the occurrence of rapid, jerky, and binocularly symmetrical eye movements (Aserinsky & Kleitman, 1953; Dement & Kleitman, 1957) and was later described as an independent electrophysiological sleep state with muscle atonia by Jouvet et al. (1959). This rapid eye movement sleep (REMS) was deemed paradoxical by Michel Jouvet because it was marked by a brain activity similar to what is recorded during wakefulness in a sleeping body. Other than the ocular saccades and the muscle atonia, this state physiology also features a very characteristic muscle atonia, a deregulation of the cardiac and respiratory frequencies (Snyder et al., 1964), a loss of thermoregulation associated with a significant increase of the brain temperature and other peripheral traits (e.g., pilo- and penile erection, shivering) (Parmeggiani, 2003; Schmidt et al., 1994). In humans, REMS is also thought to be

the vault of dreams (Dement & Wolpert, 1958; Roffwarg et al., 1966). Dreamlike behaviour has been identified in various mammals and associated with REMS. Those behaviours were defined based on the appearance of twitches of the limbs, tail, and face. In humans, those jerky motor automatisms were correlated to dream content and their re-enactment, which was notably visible in patient with neurodegenerative pathologies (Arnulf, 2012). This co-occurrence of dreams and their re-enactment was thought to be the reason for the deep muscle atonia, to protect the individuals as well as the state itself (Valencia Garcia, Luppi, et al., 2018) but is now less obvious with report of dream occurring in SWS (Siclari et al., 2017; Stickgold et al., 2001).

Due to its particularly active phenotype for a sleep state, REMS has drawn a lot of interest over time trying to understand its function(s). A lot of different hypothesis have been proposed since the discovery of REMS but most failed to meet a consensus or were rejected based on counterarguments found in the animal kingdom (for review see Blumberg et al., 2020; Schmidt, 2014). For example, one of those longstanding theory was that sleep is made to conserve energy (Berger & Phillips, 1995). Sleep was seen as a passive phenomenon in which all physiological processes were reduced much like what happen during torpor or hibernation. However, as we just saw, REMS is anything but passive. Most of the metabolism is increased during REMS and, therefore, this state seems to contradict this model. Moreover, the comparison between the energy consumption during quiet wakefulness and sleep is relatively small and some authors argued that it is negligible for the overall energy conservation to be considered sleep's primary function (Rechtschaffen, 1998). The stronger present hypothesis is the ontogenetic theory (Blumberg, 2010; Del Rio-Bermudez & Blumberg, 2018; Roffwarg et al., 1966). It comes from the observation that REMS sleep quantities significantly decrease throughout the growth of the individual and REMS is more present in the earliest stage of life (Jouvet-Mounier et al., 1969; Scriba et al., 2013). REMS is hypothesised to be responsible for the refining of the motor skills and twitches the substrate of the self-organization of the sensorimotor system (Del Rio-Bermudez & Blumberg, 2018). The role of REMS in memory has also been of particular interest since its discovery. Studies have shown the implication of REMS in emotional memory consolidation and regulation (Boyce et al., 2016; Rosier et al., 2018).

As for the SWS functions, studies have shown that this state and its components are particularly implicated in memory consolidation. During SWS, the typical slow wave pattern (SWP) is mixed with various network oscillations (e.g., thalamic spindles, hippocampal sharp-waves (HSpW), K-complex, ...) (Buzsáki et al., 2013). In particular, the hippocampal sharp waves (see Buzsáki, 2015) are known to play a key role in spatial memory consolidation (Girardeau et al., 2009). Interestingly, it has been shown that the coupling of those network oscillations (namely the spindles, HSpW, and SW) facilitates hypothalamo-cortico-communication and memory consolidation during SWS (Maingret et al., 2016).

Overall, sleep has been shown to play key role in the recycling and synthesis of molecules (Mackiewicz et al., 2007; Xie et al., 2013), and synaptic regulation (Cirelli & Tononi, 2021; Li et al., 2017). Moreover, the global energy allocation theory put sleep at a vital position in



energy management and survival of the individuals (Schmidt, 2014). This theory proposes a universal sleep function in energy allocation and puts the impairment of thermoregulation processes during mammalian REMS at a central position for the optimisation of the energy management (Schmidt et al., 2017) making the comparison between homeotherms and poikilotherms of particular interest.

### Sleep neuroanatomy

Another approach to pinpoint the function(s) of REMS & SWS has been to try and uncover the sleep network implicated in these states and its relation to wakefulness. A lot of interesting studies have been made on the wake and sleep network and we will briefly summarize them here (see Figure 1; for more detailed review see Luppi & Fort, 2019; Peever & Fuller, 2017). The first aspect of sleep modulation is wake and its promotion. Wake-on nuclei are mainly found in the pons, use a wide variety of neurons and neurotransmitters, and are highly redundant allowing strong projections to the thalamus and the cortex. To name some of the main nuclei involved in wake, there are the locus coeruleus with noradrenergic neurons, the dorsal raphe nucleus with serotonergic neurons, the medial Parabrachial nucleus with glutamatergic neurons and the ventral tegmental area with dopaminergic neurons (Eban-Rothschild et al., 2016). The main outcome of those nuclei is their inhibition of sleep-on nuclei, like the ventrolateral-preoptic-nucleus (VLPO), the ventro-lateral periaqueductal gray (vIPAG) or the nucleus accumbens (Nac), and the processing of sensory input.

In physiological conditions, switching from wake to sleep always mean switching from wake to SWS. Studies have shown that this SWS promotion is possible thanks to the suprachiasmatic nucleus (SCN). This small sub-cortical structure is highly sensible to light and is responsible for the circadian rhythm of mammals (Moore et al., 2002). In rodents and other nocturnal species, light means the activation of the SCN that in turn activates the VLPO. The VLPO is mainly compose of GABAergic inhibitory neurons and, when activated by the SCN, this nucleus strongly inhibits the wake-on nuclei, inducing sleep (Sherin et al., 1996). This inhibition lifts the inhibition of the reticular thalamic nucleus (RT). It has been shown that those GABAergic neurons played a key role in the generation of SWS and its maintenance but also in the generation of the SWP and spindles (Halassa et al., 2011). It was also shown that the parafacial zone might be implicated in the induction of sleep. Those GABAergic neurons inhibits the glutamatergic neurons of the medial parabrachial nucleus (MBP) lifting their excitatory effect on the basal forebrain (BF) (Anaclet et al., 2012, 2014). As for the transition from SWS to REMS, a lot is yet to be uncovered on what might drive this transition. However, when first discovered, it has been demonstrated that REMS is generated in the pons (Jouvet & Michel, 1959). Later studies showed that the glutamatergic neurons of the sublatero-dorsal tegmental nucleus (SLD) are drivers of REMS (Jouvet, 1962). This structure is under the inhibition of the ventro-lateral periaqueductal gray (vIPAG) and could not play its role without the intervention of the MCH neurons of the lateral hypothalamus that lift this inhibition by inhibiting the vIPAG (Luppi & Fort, 2019). When activated, the SLD activates the ventral alpha-gigantocellular reticular nucleus (GiV) and its glycinergic and GABAergic neurons that are responsible for the deep muscle atonia during

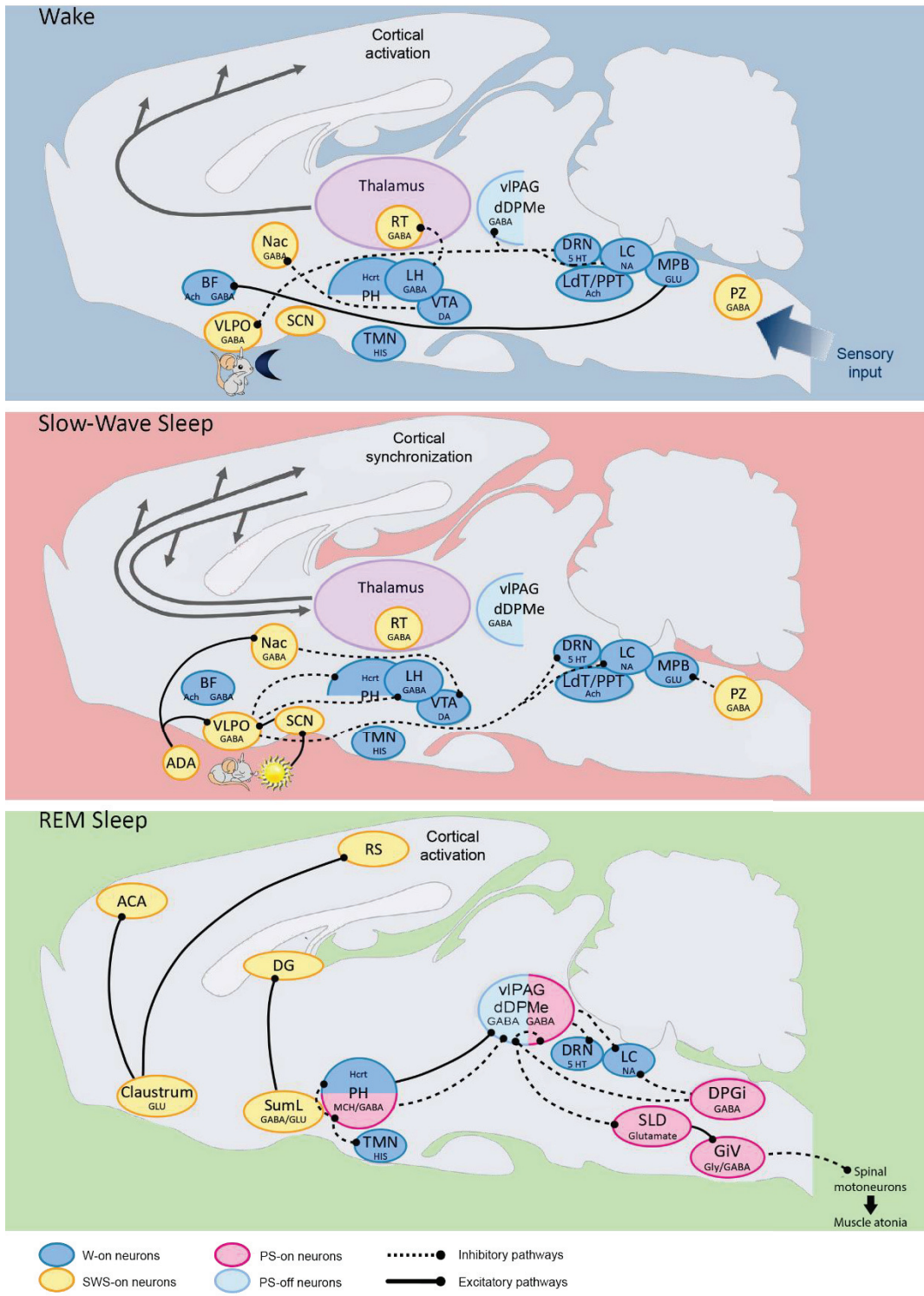


Figure 1 – A. Classical neuronal networks responsible for wake. B. Classical neuronal networks for SWS. C. Classical neuronal networks responsible for REMS. Extracted from Luppi & Fort, 2019.

5HT, 5-hydroxytryptamine (serotonin); Ach, acetylcholine; ADA, adenosine; BF, basal forebrain; DPGi, dorsal paragigantocellular reticular nucleus; dDPMe, deep mesencephalic reticular nucleus; DRN, dorsal raphe nucleus; GABA, gamma-aminobutyric acid; GiV, ventral gigantocellular reticular nucleus; Gly, glycine; Hcrt, hypocretin (orexin)-containing neurons; His, histamine; LC, locus coeruleus; LdT, laterodorsal tegmental nucleus; MCH, melanin concentrating hormone-containing neurons; MPB, medial parabrachial nucleus; NA, noradrenaline; Nac, nucleus accumbens; PH, posterior hypothalamus; PPT, pedunculo-pontine tegmental nucleus; PS, paradoxical sleep; PZ, parafacial zone; RT, reticular thalamic neurons; SCN, suprachiasmatic nucleus; SLD, sublaterodorsal nucleus; SWS, slow-wave sleep; TMN, tuberomammillary nucleus; vIPAG, ventrolateral periaqueductal gray; VLPO, ventrolateral preoptic nucleus; W, wake.

REMS (Valencia Garcia, Brischoux, et al., 2018; Valencia Garcia, Luppi, et al., 2018). In turns, cholinergic and non-cholinergic neurons of the SLD and other nuclei projects to the thalamus and hypothalamus playing a key role in the cortical activation of REMS. It has been demonstrated that the supramammillary nucleus (SuM) activates the dentate gyrus and the claustrum activates the other cortical structures during REMS (Renouard et al., 2015). While the function of the dentate gyrus is quite well documented, the role of the claustrum remains a mystery (Amaral et al., 2007; Crick & Koch, 2005; Kesner, 2013). This thin layer of neurons stuck between the insula and the striatum, in mammals, is reciprocally connected to almost all cortical areas. Proposed as the 'seat of consciousness', it seems to also be involved in the segmentation of attention (Goll et al., 2015), and thus REMS. Yet, the relation between those nuclei and the other structures generating REMS remain to be uncovered.

Still, even if great advancement has been made on sleep networks and their potential role in sleep function(s), no unifying or universal theory has been fully verified and sleep and its duality remain a mystery as for why and how it happens.

### Sleep in the animal kingdom

One critique that can be made on the functional and neuroanatomical studies in sleep is that, for the vast majority of them, they lack in diversity. Most of the recent and historical studies on sleep have been made on a reduce set of mammalian species (i.e., humans, cats, and rodents). They have allowed unprecedented improvement in our understanding of sleep and demonstrated a fine homogeneity between those "lab" species. However, studies interested in non-lab models have shown that the story might not be that simple.

Here, we wanted to briefly review how sleep and its states could be expressed in non-conventional species to get a better grasp of what sleep might be.

### Mammals

In mammals, the first key differences between species are the quantities of each sleep state. The length of sleep varies greatly across species with some sleeping only a few hours (e.g., donkeys (*Equus asinus*) sleeping 3 hours per day (Ruckebusch, 1963) or giraffes (*Giraffa camelopardalis*) that sleep 4h30 per day (Tobler & Schwierin, 1996)) to others sleeping for most of the day (e.g., southern opossums (*Didelphis marsupialis*) sleeping more than 19h a day (Van Twyver & Allison, 1970) or the armadillo (*Chaetophractus villosus*) that sleeps almost 20h a day (Affanni et al., 2001)). These sleep quantities also impact the quantities of REMS with species experiencing less than 15 minutes of REMS per day. Like we discussed earlier, SWS and REMS play key roles in memory processing. Does this mean those species do not possess the same cognitive abilities than others with more time spent sleeping? Apparently no, one notable example is the one of elephant matriarchs that showed in the wild very little sleep and rare to no period of REMS-like behaviour for days without compensatory behaviour whatsoever (Gravett et al., 2017). Those animals are often praised for their lifelong mnemonic abilities and complex cognitive skills (Plotnik et al., 2011), and the very little time spent sleeping tend to suggest they might have developed compensatory mechanisms.

So far, we have only talked about land mammals, but some mammals spend a significant portion, if not all, their lives in water. Studies on cetaceans (e.g., whales, dolphins, beluga, ...) and pinnipeds (e.g., walrus, seals, sea lions, ...) have shown that those species do not express REMS behaviour when at sea (Lyamin et al., 2013), suggesting that fully aquatic mammals might not express REMS. Moreover, those aquatic species display an unusual behaviour, unilateral SWS (USWS). Reports in cetaceans and fur seals have shown that these species could have a continuous activity and still get some rest by sleeping in only one brain hemisphere while the other stays awake, aware of its environment and able to react accordingly (Lyamin et al., 2016; Mukhametov et al., 1977). This unique behaviour is thought to be useful in continuous swimming conditions allowing them to come up at the surface to breath regularly or rest while continuously moving in an environment with high predatory pressure.

However, USWS is not the only variant of sleep present in mammals. Monotremes, the “basal” group of mammals, is also quite unique in its expression of sleep. Studies in platypuses and echidna have shown that these species display a mixed sleep state sharing common components of REMS and SWS (i.e. SWP associated with REMS behaviour, Siegel et al., 1996, 1999). Other groups of species show traits that are not consistent with the commonly accepted mammalian criterion of the two sleep states. Armadillos (*Chaetophractus villosus*) display penile erection during SWS and not REMS like most mammals (Affanni et al., 2001; Schmidt & Schmidt, 2004), blind moles rats (*Spalax ehrenbergi*) do not display rapid eye movements (REM) during REMS (Tobler & Deboer, 2001), ... If the latter is probably due to the regression of the visual tracts of the species, it is still quite unclear why the armadillos do not behave like the rest of the mammals.

## Birds

Birds have, like mammals, colonised every continent of our planet and they come in various form, size, and colours. It is estimated that there is twice as many species of birds than there are mammals. Their sleep behaviour is surprisingly similar to what we observe in mammals. Birds possess a neocortical architecture quite different from the six-layered neocortex of mammals (discussed later). However, they display the two same sleep states as mammals. The existence of some of the SWS components are still discussed today, however, (Payne et al., 2020; van der Meij et al., 2019) and their REMS episodes are a lot shorter than in mammals (for review see Rattenborg, 2006). One major difference between bird sleep and that of mammals is the relative absence of atonia during REMS. Birds have the ability to stay perched, sometimes even only on one foot, throughout their REMS episodes. They do, however, show some form of muscle atonia in their neck muscle and that phenomenon is most visible in species with long necks, like geese (Dewasmes et al., 1985). This phenomenon is probably not due to a passive leg locking mechanism, like in horses, but could be due to bird’s ability to retain some muscle tone even during REMS (Rattenborg et al., 2019). Like in mammals, even if most birds meet the classical features of the two sleep states, some have shown different variants of the states.

Owls, for a start, display all signs of SWS and REMS alternating between the high amplitude slow wave of the SWS and the desynchronised electroencephalogram (EEG) activation of REMS (Scriba et al., 2013; Šušić & Kovačević, 1973). They also show behavioural signs of REMS like the partial atonia translated in their head dropping or twitches, but they do not display REM during those episodes. This lack of eye movements is probably due to their visual system anatomy because their large eyes are almost locked in place in their eye sockets (Plochocki et al., 2018).

Great frigatebirds (*Frigata minor*) also display some plasticity in their sleep phenotype. These birds can drastically reduce their SWS and REMS quantities (less than 10% compared to their on-land sleep) for days to forage for food. Even more surprising, they can sleep while flying using USWS and air currents. Using upwards air currents, they are able to sleep in one hemisphere, and even sometimes both, while continuously flying and gliding on the currents (Rattenborg et al., 2016).

But continuous flight is not the only occurrence where birds can modify transiently their sleep phenotype. Male pectoral sandpipers (*Calidris melanotos*) can suppress their sleep during mating periods to increase their fitness. By being more active during mating season, they are able to mate with more females and increase the number of offspring they have, showing that suppressing sleep does not always mean diminishing your performance (Lesku et al., 2012). Mallards (*Anas platyrhynchos*) also use USWS but in an anti-predatory context. When on land, in a group, mallards at the group periphery tend to use USWS and keep their eye looking outside the group open and the contra-lateral hemisphere awoken, staying vigilant to potential external threats (Rattenborg et al., 1999).

Like monotremes in mammals, palaeognaths, the “basal” birds, (e.g., ostriches, emu, ...) seem to display what appears as a hybrid sleep state fusing SWS brain patterns with REMS behaviours. Ostriches still show signs of REMS with their head dropping, eye movements, but the brain activity is characterised by high amplitude slow waves (Lesku et al., 2011). One other remarkable trait of their sleep is the length of those REMS. The episodes are notably longer than the ones recorded in other birds (up to 5 min) and they spend more time in REMS than any other bird (24% of sleeping behaviour). A recent study reported the same mixed state in ostrich chicks. In addition, even if it is disputable, they also reported that the chicks spend significantly less time in REMS than adults, leading the author to suggest that the ostriches did not follow the ontogenetic theory (Lyamin et al., 2020). However, this mixed state seems to be only present in a specific part of the paleognaths as one of their representative, the elegant crested tinamou (*Eudromia elegans*), does not present this mixed state and shares the same two patterns as the rest of the birds (Tisdale et al., 2017).

#### Non-avian reptiles

Reptiles is a commonly misused term to talk about a paraphyletic group of species that are the amniote relatives of birds and mammals. It actually describes the monophyletic group containing the birds and three groups of species, the crocodylians (alligators, crocodiles, and caimans), the chelonians (turtles and tortoises) and the lepidosaurians (lizards, snakes,

tuataras, ...) that are poikilotherms species contrary to mammals and birds, meaning that their body temperature is variable relative to environmental conditions. Therefore, to talk about the 3 poikilotherm groups and avoid miscommunication in this manuscript, we will refer to this group as non-avian reptiles, explicitly excluding the birds. That said, sleep in those species is very poorly characterised and no real consensus is available on their sleep phenotype so far (for review see Libourel & Herrel, 2016).

### Crocodylians

Crocodylians are the closest relatives to birds being part of the archosaurs. They are the smallest group of extant reptiles with only 24 species remaining. The number of studies that took interest in their sleep is even lower (6 studies to date) and were all made over forty years ago. However, those studies have demonstrated that behavioural sleep does exist in crocodylians. They appear to have a quiescent state during their nycthemeral period with increased arousal and a distinct brain pattern with higher amplitude and lower frequencies compared to wake. However, the studied species did not appear to show signs of REMS (either in the brain activity or their behaviour). Only one study on one individual of *Caiman latirostris* reported very brief (less than a minute) and rare (~ 9 per nights) periods with twitches and eye movements (Peyrethon & Dusan-Peyrethon, 1969). The existence of REMS could not, however, be excluded in some studies due to the non-continuous recording methods.

### Testudines

Turtles and tortoises are also largely understudied with only 11 studies on 9 species out of the more than 350 species existing. This group is notably interesting because it contains fully aquatic species (turtles) and fully terrestrial ones (tortoises). Studies on terrestrial species did not manage to clearly establish the existence of two distinct brain activity during sleep. Some studies reported the appearance of high voltage spikes during the quiescent state leading on the idea that this spiking activity could be a correlate of sleep activity (Ayala-Guerrero, 1987; Ayala-Guerrero et al., 1988; Flanigan, 1974; Walker & Berger, 1973). Moreover, pharmaceutical experiments showed that in tortoise the spike rates increased after injection of sodium pentobarbital (Hartse & Rechtschaffen, 1982). The authors showed that the tortoise spikes responded the same way as the hippocampal sharp waves did in cats. They concluded that those results reinforced the idea that those spikes were electrophysiological signs of sleep and propose them as analogous to the mammalian hippocampal sharp waves. Furthermore, some studies reported a potential behavioural REMS like state. This possible sleep state was mostly due to reports of motor automatisms and were most of the time accompanied with an increase in heart rate (HR) (Ayala-Guerrero, 1987; Ayala-Guerrero et al., 1988; Vasilescu, 1970). However, like any other study in non-avian reptiles, no author was able to differentiate those short episodes of REMS like states with short awakening. The only study on an aquatic species, sea turtle (*Caretta caretta*), however, did not report any behavioural signs of a sleep dichotomy or even electrophysiological signs of sleep in this

species suggesting that this species never slept (Šušić, 1972). Yet, this report should be considered with caution, the authors reported quiescent periods that partially match our definition of a sleeping behaviour and while the author did try to induce sleep deprivation the length and conditions of this experiment were not sufficient to properly assess potential sleep homeostasis. Still, this report is the only available report on a fully aquatic non-avian reptile and show that sleep and its behaviour might have been drastically impacted by evolution in this unique environment.

Sleep in testudines is unclear. The reports are, like in crocodylians, lacking some crucial information and were made almost half a century ago. Nonetheless, sleep seems to be characterised in testudines by the occurrence of arrhythmic isolated spikes or in burst and a division might be made between two behavioural sleeps based on presence of jerky motor automatisms even if we cannot exclude that those movements could be marks of short awakenings.

### Squamates

Squamates contain a wide diversity of species with a diversity in lifestyle, diet, ... They are the most studied group of non-avian reptiles for sleep with 12 recorded species (Libourel & Herrel, 2016) mainly in the 70's. Based on arousal threshold and sleep deprivation experiments, those papers were able to demonstrate that lizards were behaviourally sleeping. In addition, half of them also proposed the existence of two sleep states. the possible REMS state was based on a wake like EEG and the co-occurrence of ocular and sporadic movements of the extremities. However, like in other REM sleep reports in reptiles the differentiation with short awakening could not be rejected.

More recently, papers with detailed measures on the physiology and brain activity during sleep behaviour were published on the bearded dragon (*Pogona vitticeps*) (Norimoto et al., 2020; Shein-Idelson et al., 2016) and on the Argentine tegu (*Salvator merianae*) (Libourel et al 2018). Those papers challenged the classical view of lizard sleep. In Chapter I, we will discuss in depth the existing data about sleep in those species and its implication for sleep research in this published review.

### Sleep and Comparative Neuroanatomy

We talked about neuronal networks and their implication in sleep in mammals. We also talked about the pertinence to compare apples and oranges when trying to apply mammalian sleep criterion to the rest of the animal kingdom. In this paragraph, we want to take a look on what is known about brain architecture and the correlates between structures in mammals, birds, and non-avian reptiles. The major difference between those groups is their cortical architecture. Mammals have a thick 6 layered cortex with tangential expansion, birds do not have laminated brain but have a cortex organised in nuclear slabs or wulsts and non-avian reptiles show a thin three-layered cortex. Do those difference in structure allow the considered species to express the diverse sleep phenotypes? When detected, REMS or REMS-like behaviour is characterized by a desynchronised activation of the cortex similar to

wakefulness (except for the Argentine tegu) and is achievable in each of the three cortical configurations. SWS however, and SW, are highly dependent of the cortical architecture and its reciprocal connectivity with the thalamus (corticothalamic connections) and within itself (corticocortical, or palliopallial, connexion). It has been hypothesised that whereas mammals and birds show extensive palliopallial connectivity, sufficient for generating SWP detectable by EEG techniques, non-avian reptiles lack those interpallial connections and are unable to produce sustained SWP (Rattenborg, 2006). Yet, a transcriptomic study showed that the sharp waves reported in the lizard, *Pogona vitticeps*, were generated in what has been identified as the non-avian claustrum (Norimoto et al., 2020). Those waves in turn propagate to the rest of the pallial structures (and in particular the DVR) and lesions of the claustrum suppressed them. The relation of the claustrum and the DVR could be of particular interest to understand sleep and its generation in squamates and other non-avian reptiles. As shown in figure 2, the DVR is thought to be part of the neocortex of the non-avian reptiles and could be an equivalent of the amygdala and sensory processing areas (Tosches et al., 2018). A lot is yet to be learned on the generation of SWP in non-avian reptiles, but the non-avian claustrum and the rest of the pallium could help unlock some answers.

As for the rest of the sleep network discussed previously, very little is known in non-avian reptiles about the activity of those structures during sleep. As evoked in the review, the thalamus, pons, and medulla are well conserved throughout evolution as well as their neuronal populations (Ayala-Guerrero & Mexicano, 2008b; Cardot et al., 1994; Domínguez et al., 2010; Hoogland, 1982; Wolters et al., 1984) and their relations, especially to the claustrum (Norimoto et al., 2020). Thus, their connectivity is probably highly similar in all amniotes. Still,

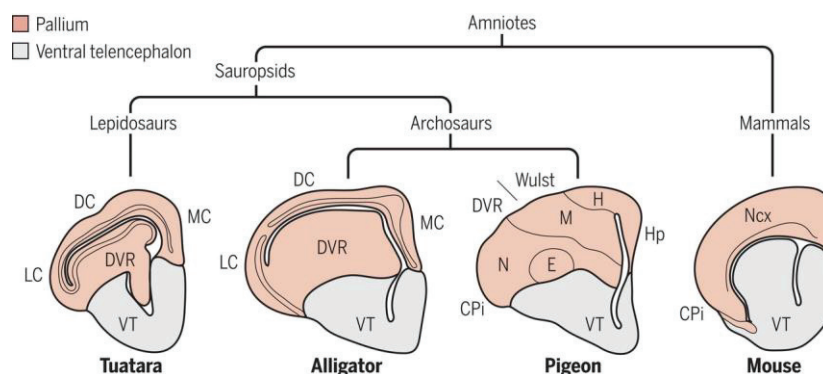


Figure 2 – Schematic representation of the pallium evolution across the amniotes. Every amniotes brain can be divided into two parts, the ventral telencephalon (VT, grey shading) and a dorsal telencephalon (i.e. pallium, peach shading). Mammals pallium is composed of the neocortex (Ncx), the piriform cortex (CPI), and the hippocampus and the amygdala (not shown). Reptiles pallium (birds and non-avian ones) can be divided into two, the dorsal ventricular ridge (DVR) and their cortex. In birds, the DVR contains the ventral part of the mesopallium (M), the nidopallium (N), the entopallium (E), and the arcopallium (not shown). Birds have a medial hippocampus (Hp), a lateral piriform cortex (CPI), and a dorsally located Wulst that includes the dorsal mesopallium and the hyperpallium (H). In non-avian reptiles, the cortex can be divided into three parts, lateral (LC), dorsal (DC), and medial cortex (MC). Drawings are not to scale. Extracted from Briscoe et al., 2018.

a great deal of work remains to be done to uncover the sleep neuronal network dynamics in non-avian reptiles.



## PhD strategy and objectives

The aim of this PhD work is to try and lift part of the veil shrouding sleep, its states, and their origin. In the first part of the PhD, I will review and discuss in detail the recent discoveries on sleep in the bearded dragon and in the argentine tegu. This part will show that our knowledge on sleep in reptiles raise many questions about the nature of a possible REMS in reptile and questioning the potential homology. In a second chapter, I will describe in detail sleep from a behavioural physiological and cerebral point of view in three different lizard species to provide more insight into the diversity of the sleep and its expression in reptiles.

## Terminology

To avoid miscommunication and detrimental shortcut in our terminology, we wanted to address some of the term we are going to use in this manuscript. The first one concerns the high amplitude waves described in *Pogona vitticeps* and some species of non-avian reptiles. At first, they were dubbed Sharp Waves (ShW) for their morphological resemblance with the Hippocampal Sharp-Waves recorded in mammals and were thought to be the equivalent of the mammalian SWP for lizards. However, it is increasingly clear that they are neither generated in the hippocampal equivalent of the non-avian reptiles, their medial cortex, nor their thalamic region because, as we saw, they do originate, in the bearded dragon, from their proposed claustrum in the anterior part of the DVR (Norimoto et al., 2020). Thus, to avoid inference on their function or their origin, we will discuss those type of high voltage waves as High voltage Waves (HvW).

Secondly, we wanted to discuss our sleep state terminology. As we just saw in this introduction, the lab mammalian criteria for SWS and REMS are not set in stone and can fluctuate in the animal kingdom. To avoid mislabelling our states, and as long as clear structural and functional parallels are not made with their mammalian counterparts, we are going to describe and characterise the sleep states of our lizards as sleep one (S1) and, if needed, sleep two (S2). S1 would be the “quiet” sleep state (proposed to be homologous to SWS in the bearded dragon) that followed wakefulness and S2 a state sharing partial similarity with mammalian REMS.

Lastly, when discussing brain activity, one can hardly avoid talking about frequency bands. Those bands are used to describe the brain spectral power at different frequencies. It is now well known that temperature has a significant impact on the power of those bands even in homeothermic species (Deboer, 1998; Ten Cate et al., 1949). It becomes even more critical when talking about band power in poikilotherm species. Thus, in this manuscript, we are going to use the current delimitation of those bands (i.e.,  $\delta$  (0.5-4 Hz),  $\theta$  (4-8 Hz),  $\alpha$  (8-12 Hz),  $\beta$  (12-30 Hz), low  $\gamma$  (30-100 Hz)) but without any inference of their function or their correlation to mammalian or avian equivalents due to the potential impact of temperature variations.

# Chapter I – Is there REM sleep in reptiles? A key question, but still unanswered.

Paul-Antoine Libourel and Baptiste Barrillot

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## Bullet points:

- A third of the papers on sleep in reptiles reported a REM sleep-like state.
- However, none of these studies provides enough clues to postulate a true homology with mammalian REM sleep.
- As reptiles are anatomically and physiologically not capable of expressing all the traits that define REM sleep in mammals (and probably birds), the quest to identify REM sleep in reptiles, based on all its mammalian traits, could be unfounded.
- The origin of REM sleep will be revealed through more comparative studies focused on how animals sleep, rather than whether they have REM sleep, as defined in mammals.

## Keywords:

Reptiles, REM sleep, bearded dragon, Argentine tegu, sleep

## Abstract:

REM sleep was initially described in mammals and was later found in birds. As the results obtained in reptiles were unconvincing, this suggested for years that only homeothermic species have REM sleep. However, recent studies in lizards also suggest the presence of two electrophysiological states and provide a better picture of their sleep. Nevertheless, due to differences in the expression of these states in two lizard species, as well as missing information, homology with mammalian REM sleep still could not be established. As ectotherms and homeotherms have very different physiology and neuroanatomy, we expect that, even if a state like REM sleep exists in ectotherms, its phenotypic expression should be different. Therefore, we think that a comprehensive understanding of the evolution of REM sleep will come from efforts to examine both the similarities and difference between sleep states in homeotherms and ectotherms.

## Introduction

Sleep is a ubiquitous phenomenon found throughout the animal kingdom (Nath et al., 2017). However, the division of sleep into two distinct sleep states (non-rapid eye movement (NREM) sleep and REM sleep) was often thought to be the monopoly of the homeothermic species (mammals and birds) (Kavanau, 2002). Recent discoveries (and often forgotten old papers), however, challenged this assumption (Leung et al., 2019; Libourel et al., 2018; Shein-Idelson et al., 2016). Besides the factual and evolutionary interest in knowing that REM sleep could be much older than expected if it is not only found exclusively in “warm blooded” species, its existence in ectothermic animals, would drastically impact our view of its function(s); notably, by rejecting the hypothesis that REM sleep coevolved with thermal genesis. This would suggest that REM sleep was not correlated with the active lifestyle of homeotherms. As the function(s) of REM sleep remains unknown, its presence or absence in “cold blooded” species is crucial to determine why it exists. Therefore, this question should be addressed with the utmost care and effort to properly assess its evolution and existence across species.

*What is REM sleep? A mammalian and avian view:* Before screening ectotherms for REM sleep, it is necessary to first think about what REM sleep is. Its discovery in humans and cats in the fifties was made based on the observation that eyes exhibit rapid saccades during behavioral sleep (Aserinsky & Kleitman, 1953; Dement & Kleitman, 1957). The activated wake-like brain activity measured via electroencephalography (EEG) during REM sleep is also one of its major features that gave REM sleep another name: paradoxical sleep (Jouvet et al., 1959). During REM sleep, many physiological parameters change dramatically. The cardiac rhythm loses its regularity (Snyder et al., 1964), motor automatism and twitches are frequent, thermoregulation mechanisms are turned off (Parmeggiani, 2003), penile erection occurs (Schmidt et al., 1994), brain temperature increases (Parmeggiani et al., 1984), and the cerebral blood flow also increases (Bergel et al., 2018). All of these phenomena occur while a deep muscle atonia is present in most skeletal muscle (Jouvet & Michel, 1959). Since its discovery, multiple species have been screened to determine if they also express REM sleep. Based on this definition (often simplified as all the parameters were not easily accessible) a REM sleep-like state, with a wake-like EEG, rapid eye movements, and sometimes muscle atonia and muscular twitches was identified in all terrestrial mammals screened to date, as well as in all birds examined (Campbell & Tobler, 1984).

*REM sleep in Reptiles: what was found in the 70's:* REM sleep seems to be expressed in a similar way in mammals and birds, but what about reptiles (lizards, snakes, turtles, and crocodiles) that share a common ancestor with their homeothermic cousins? Do they express this phenomenon? This question was investigated as soon as REM sleep was discovered. Most of all the experiments were conducted on these taxa before the 80's; but unfortunately, those experiments failed to reach a consensus on whether reptiles exhibit REM sleep (reviewed in Libourel & Herrel, 2016). Indeed, as illustrated in Figure 3, from those old papers around half of the studies in lizards (11 of 20 studies) reported the presence of two sleep states suggesting the existence of a putative REM sleep state (Libourel & Herrel, 2016). Three of the 12 papers conducted on turtles and one out of the 6 on crocodylians species reported two sleep states (Libourel & Herrel, 2016). Then, since the 60's, two sleep states were already reported in

reptiles, however their nature still remain unclear. This is partially explained because the EEG findings vary greatly across studies, sometimes even in the same species.

The findings include either no electrophysiological difference between wake and sleep, signs of one NREM sleep-like state, or two types of sleep suggestive of NREM and REM sleep (reviewed in Libourel & Herrel, 2016). Slow waves have rarely been reported in reptiles but isolated high-voltage sharp-waves (HShw) were often proposed as a marker of sleep, similar to the mammalian hippocampus Sharp waves or cortical slow waves (Hartse, 1994; Libourel & Herrel, 2016; Tisdale et al., 2018).

In reptiles, most of the studies, claiming that REM sleep (or at least a similar state to REM sleep) was present, based their conclusions on the presence of a wake-like EEG during behavioral sleep, associated with eye movements and sometimes motor automatism, seen in its mammal counterpart. In studies reporting the presence of REM sleep in reptiles, no clear

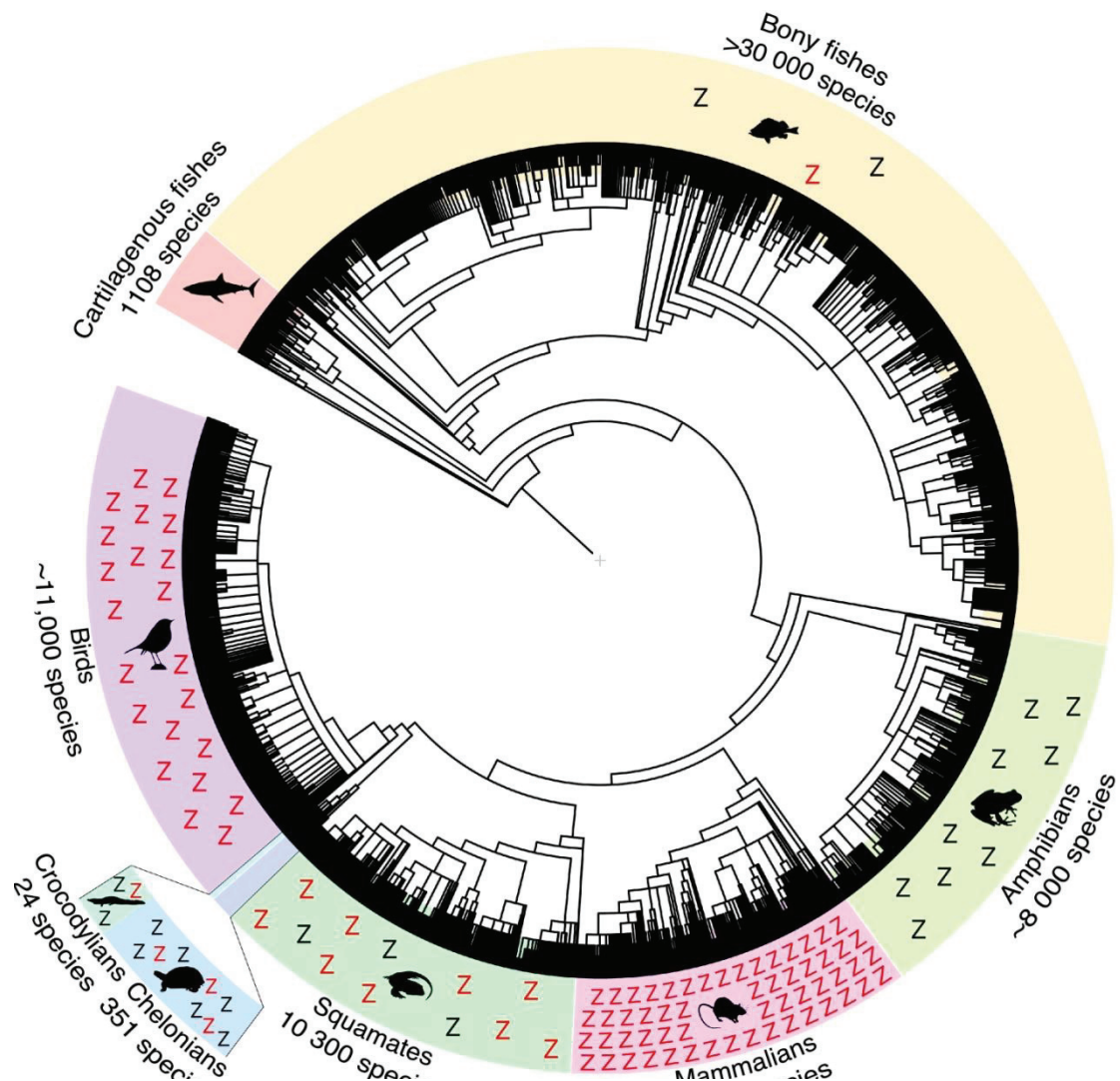


Figure 3 – Phylogenetic tree of the Chordata displaying the number of species studied per family; the Z icons represent the number of species recorded for sleep with electrophysiological recordings in each group. The icon is black (Z) when only one state was reported and red (Z) when two sleeps states were reported. The number of species studied is based on (Capellini et al., 2008; Libourel & Herrel, 2016; Roth et al., 2006). The tree was built using the ‘interactive Tree Of Life (iTOL) v4’ (Letunic & Bork, 2019).

muscle atonia was recorded (only four studies reported atonia over 40 (Huntley et al., 1977; Peyrethon & Dusan-Peyrethon, 1969; Rechtschaffen et al., 1968; Vasilescu, 1970)) and only one arousal threshold evaluation during putative REM sleep was conducted (Ayala-Guerrero & Mexicano, 2008a). Thus, none of these studies were able to provide enough clues to unambiguously assert that those periods of wake-like EEG with eye movements were not short awakenings from sleep. Moreover, different brain regions were recorded, the number of individuals studied was usually small, and the ambient temperature varied across studies. All these factors might contribute to the diverse findings reported in reptiles.

*Recent discoveries:* The topic of REM sleep evolution recently returned to the main stage with two publications re-investigating sleep in reptiles (Libourel et al., 2018; Shein-Idelson et al., 2016) with up-to-date methods. Indeed, most of the previous recording were conducted with a polygraph, with data collected on paper. Consequently, it was difficult or impossible to apply signal processing analysis on the whole data set. Now, data digitally sampled at a high rate simultaneously from multiple brain regions and can be subject to spectral analysis across the entire recording. Digital video can also be analyzed precisely. In 2016, using modern methods silicon probes to record multisite local field potentials and unit activity combined with close up videos, Shein-Idelson et al. revealed that the bearded dragon (*Pogona vitticeps*) displays a period of behavioral sleep characterized by the occurrence of high amplitude sharp waves in a deep brain structure, the dorsal ventricular ridge (DVR), a possible homologue of the mammalian isocortex, or claustrum-amygdala complex (Aboitiz, 1999; Tosches et al., 2018) (discuss below). Those periods alternated with periods of desynchronized brain activity, similar to that recorded during wakefulness in these animals (Shein-Idelson et al., 2016). The periods with desynchronized activity were preferentially associated with eye movements (figure 2). The two electrophysiological sleep states alternated with an almost clock-like periodicity of 80 seconds, never recorded before in any species (including mammals and birds). The authors concluded that those two brain activities during the sleep period of the animal were the reptile equivalent of mammalian and avian NREM and REM sleep. They also suggest that the high voltage waves found could be homologous to the hippocampal sharp waves ripple complex, an electrophysiological marker of memory processing in mammals (Buzsáki, 2015). However, as explained later, these sleep homologies are still unclear.

In 2018, we replicated those results in the bearded dragon (Libourel et al., 2018). Although we found similar electrophysiological patterns during behavioral sleep, when we investigated sleep in a different lizard species, the Argentine tegu (*Salvator merianae*), those results painted a different story. Two sleep states were also identified in recordings from the same brain region (DVR) as in the bearded dragons. The first sleep state was characterized by the presence of isolated high amplitude waves (one every min on average) emerging from a background pattern without a dominant rhythm. The second sleep state showed an unprecedented, sustained brain oscillation at 15Hz, not seen during wake. This oscillation was associated with isolated eye movements (Figure 4), a very slight decrease in the muscle tone, and was suppressed after the injection of a serotonin reuptake inhibitor that suppressed REM sleep in mammals and birds. Thus, in both species two electrophysiological sleep states seems to exist, but as explained later, the expressions of none of these states completely matched

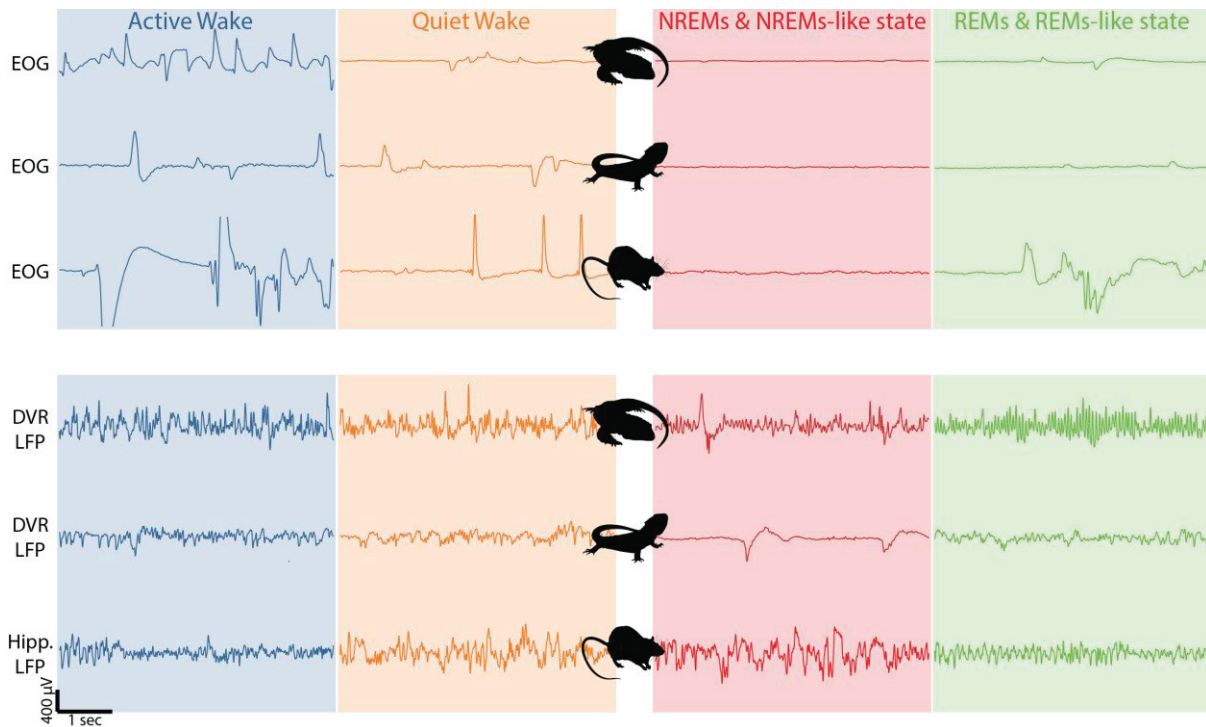


Figure 4 – Selected recordings of the different vigilant states in the Argentine tegu, the bearded dragon, and the brown rat. A. Signals of the electro-oculogram (EOG), filtered with a low pass filter at 10Hz, for Active Wake (blue), Quiet Wake (orange), and NREM and REM sleep and their putative equivalents for squamates in red and green, respectively. From the top to the bottom the species signals represented are from the Argentine tegu, *Salvator merianae* (Aa), the bearded dragon, *Pogona vitticeps* (Ab), and the brown rat, *Rattus norvegicus* (Ac). B. Signals of the local field potential for the same states and species. DVR = Dorsal Ventricular Ridge, Hipp. = Hippocampus.

classical NREM sleep and REM sleep. Therefore, we do not fully agree with the current widespread idea that reptiles have REM sleep because two states were found in two species. In the next paragraph, we will discuss the pros and cons and will argue that in a comparative context the current view of REM sleep should be challenged.

### Why the absence of consensus in sleeping reptiles should change our classical view of REM sleep?

The mammalian sleep traits used to identify or characterize REM sleep are sustained by the anatomy and the physiology of the animals. Thus, using those mammalian traits as a correlated of the presence or absence of REM sleep in reptiles, species that show important differences in telencephalic neuroanatomy, metabolism, and lifestyle, could sometimes be puzzling. Below we will review the different features that define REM sleep in mammals and birds and discuss their existence and expression in reptiles.

Reptilian brain activity during sleep states: Most of the studies that describe sleep states from cortical EEG recordings attempt to draw comparisons with what was described in mammals. However, the cortical EEG (like any brain potential) originates from the surrounding neural tissue and is therefore shaped by its neuroanatomy and connectivity with other brain regions (Buzsáki et al., 2012). But, what about species, such as reptiles, with their 3-layer cortex? This peculiar cortex seems to lack the equivalent of mammalian layer II and III, the layers with extensive intracortical projections in the mammalian neocortex (Reiner,

1993), layers that seems to play a key role in the generation of slow waves (Sanchez-Vives & McCormick, 2000). What about species that have a reduced telencephalon with a cerebellum or an optic tectum that could be more than 10 times larger than the telencephalon? Should we expect to have the same brain signature as mammals in those species? Reasonably, we might find that unlikely. Then, the absence of slow waves reported in most of the reptilian studies might simply reflect differences in neuroanatomy and connectivity.

Moreover, some reptilian studies (including the two most recent ones) recorded local field potentials (LFP) but interpreting deep brain LFP can be even more challenging than cortical EEG (figure 2). Indeed, due to their local nature, the LFP is even more variable across region. This was notably illustrated by the hippocampal sharp-waves ripples complex, a brain oscillation associated with memory processing, that occurs during immobility, and slow wave sleep in rodent (Buzsáki, 2015). The hippocampal sharp waves ripples complex can be recorded into the *stratum radiatum* layer of hippocampus CA1 region, even though the associated ripples complex shows the maximal amplitude in a different layer of CA1, the pyramidal layer (Ylinen et al., 1995) during NREM sleep. Thus, the only argument supporting homologies between the “Slow sharp” wave recorded in the DVR in the bearded dragon and the whole brain of the Argentine tegu is their morphological similarities with their mammalian counterparts. Consequently, to draw direct parallel from the LFP signature recorded in the lizards, functional and mechanistic study remains necessary. Without a proper understanding of the brain organization and the underlying neuronal networks responsible for this phenotype, the information obtained is circumstantial at best, if it is not correlated with other physiological or behavioral changes.

In addition to neuroanatomy, other factors, such as temperature, might influence brain activity signature and thereby complicate attempts to draw parallels with mammals. Indeed, even in mammals, it has been demonstrated that brain temperature influences the EEG; when brain temperature drops, EEG waves decrease in frequency and amplitude (Deboer, 1998; Ten Cate et al., 1949). In mammals, the effect is negligible as the brain temperature is kept quite constant, but in ectotherms the brain temperature follows environmental temperature. In the wild, most reptiles are diurnal and when sleeping they are exposed to colder temperature than during the day. The difference between day and night can be more than 20°C, thus, comparing the EEG in an ectothermic species under these conditions to that of mammals is questionable. Even in lab, where reptiles were often recorded in a more controlled conditions with constant temperature, around 25°C, their brain temperature is far below that of mammals. Thus, to make true comparisons, should we compare the EEG with a mammal in hypothermia or reptiles at higher temperatures? Comparisons of this sort are likely to be meaningless, as neither condition reflects normal sleeping conditions for the species. Altogether, this show how complicated it is to draw parallels between species that do not share the same neuroanatomy and body temperature.

**Sleep homeostasis:** The homeostatic regulation of sleep states is one of the major features of sleep and can be assessed through depriving animals either of all sleep, or just REM sleep. Unfortunately, in reptiles, the homeostatic regulation of putative REM sleep states has not been examined. In the Argentine tegu, after total sleep deprivation for 9 hours,

the REM sleep-like state did not increase like REM sleep in mammals. However, after this 9-hours sleep deprivation, Argentine tegu's sharp waves density increased, as reported by Flanigan in turtles, crocodiles and lizards, and like the slow waves in mammals (Flanigan, 1973, 1974; Flanigan et al., 1973) and birds (Lesku et al., 2011; Rattenborg et al., 2009). This tends to support the idea that reptilian sharp waves could be a marker of sleep intensity. However, data is missing to say whether they have the same role in memory processing or not. In the bearded dragon, Shein Idelson et al. postulated that the DVR sharp waves could be like the mammalian hippocampal Sharp Waves ripples complex. However, the fast activity observed during sharp-waves in dragons may also arise from the same processes that give rise to the fast (80-200Hz), ripple-like, activity that occurs during the up-states that give rise to extracellular slow-waves (Grenier et al., 2001). However, no functional studies have been able to confirm this homology and the only morphological similarity is not enough to draw such parallel.

The paradox of the wake-like EEG as a necessary condition of REM sleep. In the 60's, Jouvet renamed REM sleep as paradoxical sleep because of the presence of a wake-like EEG in a sleeping animal. Since this discovery, the wake-like cortical EEG has been one of the major criteria to identify REM sleep. But in 2018, we reported a sleep state in the Argentine tegu sharing similarity with mammalian REM sleep (eye movements, reduced muscle tone, suppression after fluoxetine injection), but without wake-like LFP activity in the DVR. Instead, a sustained 15Hz oscillation specific to this state was recorded. Does that mean that this electrophysiological pattern could not be REM sleep, as a distinct 15Hz oscillation was not present during wake? From our point of view, a wake-like EEG should not be a necessary criterion to identify REM sleep. Indeed, REM sleep have different functions than wake and imply the recruitment of a specific neuronal network (Luppi et al., 2011). Therefore, the electrical potentials recorded in specific brain regions should differ between wake and REM sleep. Far from the stereotype, this is also the case for rodent. Indeed, when looking carefully in rats and mice, due to the hippocampal theta rhythm, the parietal EEG during REM sleep display a very singular and regular frequency at 7 Hz. As show in figure 4, this oscillation differs from the parietal EEG of an awake animal (not walking) where the frequency is more spread through the theta band (4–9Hz).

Eye movements during sleep in reptiles? Eye movements were associated with REM sleep since its discovery, making ocular saccades one of the major signatures of REM sleep that has been used as a marker of its existence in other species. In almost all reptile's studies, from crocodiles to lizards and turtles, eye movements were reported during behavioral sleep suggesting the potential existence of REM sleep in reptiles (Libourel & Herrel, 2016). In the bearded dragon, eye movements were more frequent in the beginning of the sleep state with a wake-like LFP (Shein-Idelson et al., 2016). Whereas in the tegu the eye movements were more frequent during the 15 Hz state. But as no arousal threshold has been conducted, it remains possible that those periods were wakefulness or at least micro-arousal. Beside this, the density of eye movements as well as their amplitude during sleep raise questions. In the bearded dragon as in the Argentine tegu, eye movements during sleep are isolated, small and do not occur in burst like they do in mammals (Figure 4). This is not related to their ocular capacity, as during wake, eye movements are more frequent, faster, and larger in amplitude



(figure 2). Does that mean that REM sleep is not fully expressed in reptiles, or that this REM sleep-like state in reptiles is not homologous to mammalian REM sleep? Or that potential REM sleep functions associated with the eye movements are not as predominant in reptiles when compared to mammals? As all these possibilities could be true, more studies of eye movements during sleep in reptiles are needed.

**Phasic and tonic muscle activity during sleep state:** Muscle atonia, like ocular saccades and wake-like EEG activity, is a key feature of mammalian REM sleep. But unlike the other two features, muscle atonia differentiates REM sleep from wake. In mammals, it was previously thought that atonia prevents the animal from moving when its cortex is activated (during dreaming in human). This view is still likely, but challenged, in particular by the report of dreaming without movement during NREM sleep (Cavallero et al., 1992; Siclari et al., 2017) or the partial expression of muscle atonia in birds (Dewasmes et al., 1985; Rattenborg et al., 2019). Therefore, the presence or absence of this trait in animals during a REM sleep-like state could be key to understanding whether it serves an important function of REM sleep or if it is a mammalian epiphenomenon. In reptiles, muscle tone was often recorded, but muscle atonia was seldomly reported. Even in the Argentine tegu, the tone of the deep postural muscle of the neck, during the 15Hz oscillation, is significantly lower than during the rest of the sleep periods, the differences are very subtle and no clear atonia (like that in mammals) could be identified. This could be explained by the fact that no true muscle atonia is present during the possible homologue of REM sleep in reptiles, suggesting that in homeotherms, muscle atonia is either an epiphenomenon of REM sleep or serves a specific function related to endothermy. Another hypothesis to explain the absence of clear atonia in reptiles could be that they are always atonic during sleep. Indeed, as ectotherms, this would be consistent with their energy conservation strategy. Finally, if the muscle atonia was a core feature of the expression of mammalian and avian REM sleep, this could also mean that reptiles do not have an equivalent REM sleep that serves the same function as in mammals and birds.

Of all the traits of REM sleep, twitching is also one of the more obvious. In neonates, REM sleep is identified mainly from this prominent feature. Twitches occurring during REM sleep are thought to play an important role in brain development (Blumberg et al., 2013). Unfortunately, no clear associations between twitches and putative sleep states have been established in reptiles. Short movements of the toes and tail were reported sporadically in many studies of adult reptiles, but none (including the recent studies in the bearded dragon and the Argentine tegu) was able to clearly link those movements with any specific pattern of brain activity.

The apparent absence of a clear association between twitching and putative REM sleep states in reptiles raises several questions. Is twitching during REM sleep present only during development (*in ovo*) in reptiles as reported by Corner (Corner, 1977)? Or is REM sleep (defined as an active state with twitches) simply absent in reptiles? Does putative REM sleep observed in some reptiles serve the same functions as in mammals. Additional comparative and developmental work are needed to fully understand the true motor nature of REM sleep.

**Sleep architecture:** In mammals and birds, the respective quantities of NREM sleep and REM sleep vary across the development and across species (Figure 5). In addition, in birds, the duration of REM sleep episodes is much shorter, typically only lasting around 10s (Lesku et al., 2011). But more generally, as seen in figure 5, the quantity of REM sleep reported in adult mammals and birds is much lower than the quantity of NREM sleep. In the bearded dragon, the putative periods of REM sleep constitute 50% of the time asleep. If this state truly does reflect REM sleep, this would mean that dragons have the greater quantity of REM sleep ever seen in adults of any animal, (excluding the platypus with its mixed sleep states (Siegel et al., 1999)). This would have huge implications for our understanding of the functions of REM sleep. Indeed, such high proportions of REM sleep were only seen in neonates, supporting the developmental hypothesis of REM sleep (Del Rio-Bermudez & Blumberg, 2018; Roffwarg et al., 1966).

The other unique aspect of the bearded dragon’s sleep states is the almost perfect regularity of its sleep cycle, which is unlike that observed in any mammal or bird. Moreover, in mammals and birds, REM sleep increases in quantity progressively throughout the sleeping period. However, in the bearded dragon this is not the case.

The bearded dragon’s sleep architecture not only differs from that of mammals and birds, but it is also dramatically different from that observed in the Argentine tegu. In the tegu, putative REM sleep encompasses a far lower proportion of the time asleep, is more prominent at the beginning and at the end of the night and does not occur with clock-like

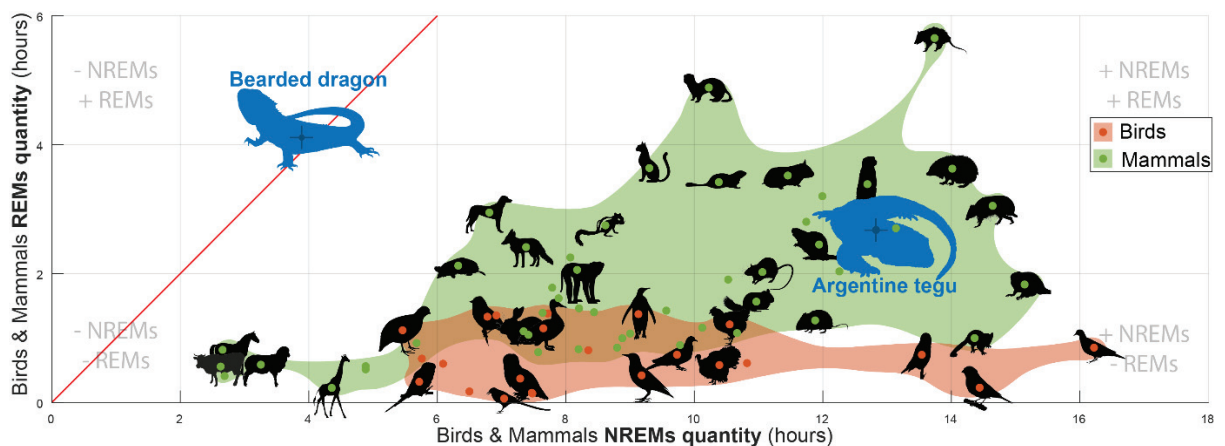


Figure 5 – Ratio of mammalian & avian REMs over NREMs compared to their putative squamates equivalents over 24h. The relative quantities of the two types of sleep for squamates are calculated based on the assumption for the NREMs-like state being an equivalent of the SWS and the REMs-like state the equivalent of paradoxical sleep (Libourel et al., 2018; Shein-Idelson et al., 2016). The relative quantities were calculated based on the results found in the literature (Capellini et al., 2008; Libourel & Herrel, 2016; Roth et al., 2006). The corners of the graph represent the minimal (-) and maximal (+) quantities of NREMs and REMs. For example, the top right corner represents the maximal quantities of both NREMs and REMs (+NREMs/+REMs).

regularity. The duration of REM bouts is also more bird-like than that of the bearded dragon. Altogether this shows how variable sleep phenotypes are, even between two species of lizards. Finally, these differences in sleep architecture between bearded dragons and mammals, birds, and tegus, underscore the challenges of determining homology between reptilian sleep states and those observed in homeotherms.

Others mammalian REM sleep features in reptiles: Aside from brain activity, muscle tone, and ocular activity, other traits have been reported during mammalian and avian REM sleep. Such traits should also be examined in reptiles to determine how similar the “reptilian REM sleep-like state” is to what has been found in mammals and birds.

One of those traits is the reduction of thermoregulatory responses in mammals and birds. However, as ectotherms, this trait would never be found in reptiles. Therefore, should we consider this feature of mammalian and avian REM sleep as a key component of this state? If the loss of thermoregulation mechanisms is considered a defining feature of REM sleep directly linked to its function, then it becomes clear that reptiles could not have REM sleep. However, this trait could also be a derived feature of REM sleep that appeared convergently in mammals and birds. Importantly, the presence of REM sleep in ectothermic reptiles, would then suggest that REM sleep had an initial function prior to its involvement in thermoregulation.

The greatest quantity of REM sleep found in young and altricial species is also a major characteristic of REM sleep, supporting its crucial role in development. Yet, even if rhythmic movements have been recorded *in ovo* in reptiles (Corner, 1977), this question has not been investigated using EEG recording.

Finally, another way to determine if REM sleep is present in reptiles would be to examine the neuronal network that generates and maintains REM sleep in mammals and birds. We now know that the circuitry that supports mammalian REM sleep involves multiples nuclei in the brainstem and hypothalamus. As the brainstem and diencephalic structures are highly conserved across vertebrates, all these neuronal populations seem to be present in reptiles (Ayala-Guerrero & Mexicano, 2008a; Butler & Hodos, 2005; Cardot et al., 1994). Therefore, it seems possible that reptiles possess the basic neuronal architecture required to generate REM sleep. However, as no precise brain atlases exists for reptiles, and despite one unsuccessful attempt to find a REM sleep specific neuronal pattern in the turtle pons (Eiland et al., 2001), the role of these structures in putative REM sleep in reptiles is unknown. The study of the activity and connectivity of reptilian structures involved in REM sleep in mammals is needed to draw a parallel and determine the level of homology between reptilian REM sleep-like and mammalian REM sleep.

## Conclusion

The phenotypic expression of REM sleep was mainly defined from mammalian studies. Most of the traits expressed in mammals during REM sleep were also found in birds, suggesting that both groups display a very similar state. As most of the mammalian features of REM sleep were found in birds (wake-like brain activity, atonia, ocular movements, twitches, homeostasis, absence of thermoregulation mechanisms, ontogenesis, architecture), it became obvious that REM sleep exists and, perhaps, supports the same function(s) in homeothermic species. In reptiles, things become more complex. Indeed, multiples studies from the 60's reported REM sleep in reptiles. Unfortunately, due to the high degree of similarity between REM sleep and wake, none of them convinced the scientific community. Recently, the studies in the bearded dragon and in the Argentine tegu nicely demonstrated

that two sustained electrophysiological patterns exist during behavioral sleep in two different lizard species. In both cases, one sleep state was preferentially associated with eye movements (often small and isolated) than the other. However, doubts remain regarding homology between these states and mammalian and avian REM sleep. Indeed, many of the other traits associated with REM sleep in mammals (e.g. complete atonia, twitches, homeostasis, loss of thermoregulation) were not found (or not fully expressed), were not investigated or could not be expressed. Further adding to the challenges of interpreting the evolutionary implications of the research on sleep in reptiles, is the finding that the expression of putative REM sleep states differs dramatically between two species of lizards studied using the same methods in the same laboratory. Therefore, it is still unclear whether reptiles have a mammalian REM sleep like state (sharing the same function) but differently expressed, if they have a partial mammalian REM sleep-like state or if they have another sleep state supporting other functions. Although our ultimate aim is to gain understanding about REM sleep in mammals, through tracing its evolution, we strongly believe that in addition to the similarities, the differences between putative REM sleep in reptiles and mammalian REM sleep should be given equal attention when studying reptiles. Indeed, because of the specific physiology and neuroanatomy of reptiles we believe that we should not expect that most of the mammalian REM sleep traits will be present in reptiles. To our point of view, the only way to understand the evolutionary history of REM sleep is to conduct more studies in additional species, and to investigate the other main traits of mammalian REM sleep, like its ontogenesis, homeostasis, neuronal basis, ... Our understanding of REM sleep will come from our emancipation from its mammalian expression. Recent very high-quality studies promote the existence of REM sleep in reptiles, and even fishes (Leung et al., 2019), seemingly pushing the origin of this state further back in time. However, we should be cautious about reaching simple clear-cut conclusions about the evolution of REM sleep that fail to incorporate the differences between species. Indeed, we strongly believe that the differences between mammals, birds, and reptiles, as well as between reptilian species, may prove to be a rich resource for examining the evolution and functions of REM sleep. This more genuine comparative approach will likely yield a more comprehensive understanding than one that attempts to force reptilian sleep states to fit within the template used to define mammalian REM sleep.

REM sleep is a phenotypic expression of a singular, mammalian state also found in birds. Just like mammals have a hairy skin and birds a feathery skin, reptiles have a scaled skin. All skins protect the animal from infection, but, in mammals and birds, it also provides a high level of thermal insulation. Just like skin, REM sleep could have different species related phenotypes and could serve common and specific functions. Therefore, we will understand its nature and functions by studying its phenotypic variability across species, not by trying to dress it in mammalian clothes.

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## Chapter II – Sleep in three species of lizards

### Introduction

As shown in chapter I, the recent study of sleep in reptiles tends to support the idea that two electrophysiological sleep states could exist in lizards. However, the full mammalian expression of these states was not completely observed. In addition, the differences between the Argentine tegu and the bearded dragon rise some reasonable doubt about the homogeneity of sleep expression across squamates. This chapter has been divided into three parts. Part I will presents new results obtained for the bearded dragon, in particular physiological changes and behavioural correlates with the two states already reported (Libourel et al., 2018; Shein-Idelson et al., 2016). In addition, those results will be compared to the phenotype expression of sleep in a sister species of the bearded dragon, the starred agama (*Stellagama stellio*). This comparison will help determine whether the unique alternance, in equal quantity of the bearded dragon's two sleep states is a singularity of this species or if this pattern has a longer evolutionary history. A second part will look at unilateral eye closure in the bearded dragon and its relation to brain activity asymmetry. Finally, as the *Agamidae* represent but a single branch of the *Squamata* tree, the third part of this chapter will look another one by characterising the physiology, behaviour, and brain electrophysiology of the Sudan plated lizard (*Broadleysaurus major*) who is part of the *Scincimorpha*. By studying this species belonging to a group never studied for sleep, this final part could unveil whether or not the origin of the two electrophysiological sleep states is more ancestral than expected and whether this alternance could be a generic phenomenon across squamates.

### Material & Methods

#### Husbandry & species

In the first study, we used four *Pogona vitticeps* (bearded dragons, 2 females) and three *Stellagama stellio* (starred agama, 3 females) aged around 2y (+/-6m), weighing 295g (+/-78g) for the bearded dragon and 117g (+/-20g) for the starred agama. In the second study, we used three *Broadleysaurus major* (Sudan plated lizards, 3 males), with an age of 2y (+/-6m), weighting 289g (+/-59). All our lizards were bought from official dealers and were maintained individually in a terrarium (1 m × 0.5 m × 0.5 m). They were fed vegetables daily and crickets thrice weekly. Water was provided *ad libitum*. Animals were maintained under a 12h:12h light/dark cycle and the room temperature was constant and set at 25°C. Each terrarium contained a shelter transparent to infrared light. Two infrared (850nm) panels (Viewpoint SA) were set on top of the terrarium and always on. All experiments were conducted according to the 3R principles in animal experimentation and in accordance with the European Community Council Directive for the use of research animals (2010/63/EEC; <https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063>). Protocols and procedures used were approved by the local ethics committee for animal experimentation of the university Lyon 1 (No. BH2017-63).

Unless explicitly stated otherwise, all animals went through the same protocol and experiments.

### Imaging

In absence of a suitable brain atlas for our considered species, and in order to target specific brain regions, anatomical MRI and CT scans were performed on the animals. Thus, prior to the surgery, all animals were sedated (half of surgical anaesthesia i.e. ketamine – 50mg/kg & medetomidine - 100µg/kg, see Supplementary II for details) and, after anaesthesia induction, the animals were placed headfirst and in supine position in a dedicated plastic bed provided with a stereotactic holder (Bruker Animal Handling Systems).

MRI acquisitions were performed in vivo on a horizontal 7T BRUKER Biospec MRI system (Bruker Biospin MRI GbmH Bruker, Germany) equipped with a set of gradients of 440 mT/m and controlled via Bruker ParaVision 5.1 workstation. A Bruker birdcage volume coil (inner diameter = 72 mm and outer diameter = 112 mm) was used for the signal transmission, and a Bruker single loop surface coil (25 mm diameter) was used for signal reception, being positioned on the head of the animal in order to target the brain. High-resolution MRI images were obtained using a 3D T2-weighted fat saturated images based on rapid acquisition with relaxation enhanced (RARE) method were obtained on an axial orientation. The acquisition parameters were as follow: Effective Echo Time (TE) 41.34 ms, Repetition Time (TR) 2000ms, RARE factor = 8, Number of Averages = 1, Bandwidth = 75kHz. The region of interest (brain) was acquired under a field of view (FOV) of 3 x 1.5 x 3 cm, and a matrix size of 386 x 192 x 128 pixels, provided an in-plane resolution of 78 x 78 x 156 µm leading to an resolution of 78 µm<sup>3</sup> after zero-filling interpolation. The total acquisition time was about 1 hour and 16 minutes. CT acquisitions were performed on an INVEON system (Siemens), with a tension of 80 kV, a current of 500 µA, an exposure time of 900 ms with 720 steps and a low magnification. The reconstruction of the final volume permits us to obtain a voxel size of 55.62 µm<sup>3</sup>. The total acquisition time was about 45 minutes.

After imaging, animals were injected with atipamezol (200µg/kg) to reverse the medetomidine effects. The MRI and CT scans were aligned using at least 10 common points between the dataset to create specific brain atlases for each species (Avizo, ThermoFisher). The coordinates of the electrode placement were determined based on those merged images (Fig. 6).

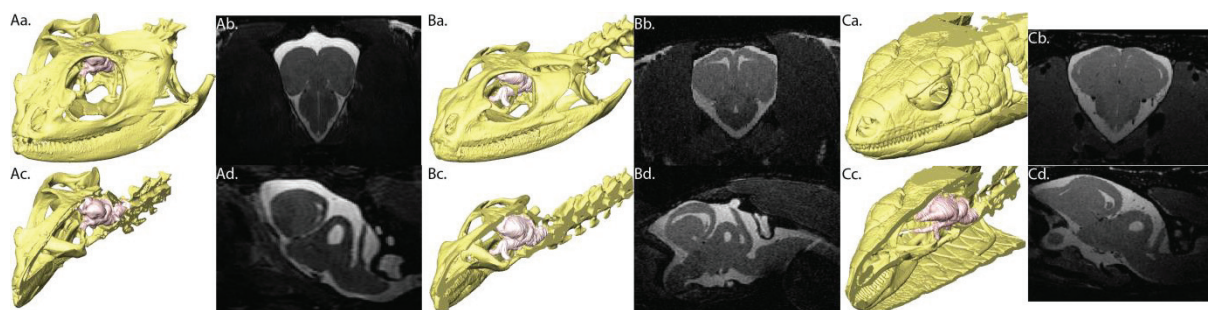


Figure 6 – Anatomical MRI and CT scan reconstructions of the brain and skulls of our 3 species of interest (A. *Pogona vitticeps*, B. *Stellagama stellio*, and C. *Broadleysaurus major*). For each species, a&c. 3D rendering of the skull (light yellow) and brain (light pink), b. MRI coronal section of the DVR, d. MRI sagittal section of the left DVR.

## Surgery

Once the implantation coordinates were determined for each animal, they underwent surgery. Surgical anaesthesia was achieved using a single dose of a ketamine and medetomidine mix injected intramuscularly (respectively 100mg/kg, 200 µg/kg, for details see Supplementary II). Reflexes and respiratory rate were checked throughout the surgery to assess a proper anaesthesia. During the surgery, two stainless steel electrodes were implanted bilaterally in the intercostal muscles to measure heart rate, and two were implanted in the neck muscles to assess muscle tone. The animals were then installed in a stereotactic frame. One screw was implanted on top of each eye on the medial part of the supraorbital ridge. Two bundles of eight 35µm diameter tungsten electrodes were implanted bilaterally in the Dorsal Ventricular Ridge (DVR). The implantation coordinates were as follows and are indicated relatively to medio-dorsal ridge of the pineal eye when visible (*Pogona vitticeps* & *Stellagama stellio*) or from the medial point between the eyes (*Broadleysaurus major*): *Pogona vitticeps* – 1.3 mm caudally +/- 2mm laterally, *Stellagama stellio* – 1.4 mm caudally +/- 1.4mm laterally, *Broadleysaurus major* – 4 mm caudally +/-1.7mm laterally. One screw was inserted on the most caudal part of the parietal bone for signal referencing and, in the *Pogona vitticeps*, a thermistance was implanted medially between the reference screw and the DVR bundles. All wires were then connected to a head connector (EIB-36-PTB Neuralynx for the *Pogona vitticeps* and the *Broadleysaurus major* & a custom-made EIB for the *Stellagama stellio*), which was secured over the skull using acrylic Superbond (Sun Medical Co.). Dental Paladur cement (Heraeus Kuzler) was applied around the head connector to protect all the wires and connector. At the end of the surgery, the animals were injected intramuscularly with atipamezol (400 µg/kg, for details see Supp #1) and subcutaneously with a mix of an anti-inflammatory solution (carprofène 1 mg/kg) and a glucose solution (5%).

## Perfusion

Under a lethal anaesthesia (twice the surgical concentrations) and after electrocoagulation lesioning (2s, 0.5 mA), animals were perfused transcardially with a 200 mL Ringer-Lactate and heparine solution (Brain Medical, France) followed by a 400 mL 4% paraformaldehyde (PFA) fixation solution, with a perfusion pump (Gilson, France) set at a rate of 20 mL/min. Brains were removed and postfixed for 2 days at 4°C in the PFA solution. Brains were included in paraffin (LEICA ASP300, Germany) and 10µm slices were cut with a microtome (Leica RM2245, Germany) for histochemical processing.

## Implant removal & Refinement

By serendipity, the removal of the implant was deemed suitable for two of the bearded dragons and the starred agamas. To locate the electrode positions *in vivo*, the *Stellagama stellio* underwent new CT scans at the end of the experiments (Libourel et al., 2018). The subsequent images were realigned to the already obtained anatomical MRI and electrode position was inferred based on the artefacts generated by the electrodes in the second scan (Fig. S3). The de-implanted animals were then placed in welcoming households.

## Recordings

### *Electrophysiology*

The behaviour of the animals was monitored with four cameras (Dragonfly2 DR2-HIBW, Point-Grey) equipped with a band-pass filter in the near-infrared wavelength. Two cameras were recording the full area, and the two others were dedicated to the animal shelter. The videos were recorded 24 h a day (VPCore2, Viewpoint), and the actimetry, which is the number of pixels changing more than 14 grey levels between two successive images, was evaluated online for each camera.

The electrophysiological signals were recorded using a custom-made wireless recording device, ONEIROS (for specifications see Massot et al., 2018). The DVR activity was recorded bilaterally using 16 tungsten electrodes at different depth (-4 to -2mm from the top of the skull). Four EOG, two EMG and two ECG were also recorded at the same time. The *Stellagama stellio* were implanted with the second version of ONEIROS which is lighter and smaller (six LFP referential recording points, two differential EOG and two differential EMG). The signals were recorded at 256 Hz with a hardware low pass filter set at 128 Hz and a high pass filter at 0.1 Hz to ensure constant recording for more than 48h with a battery weight low enough for the animals. The signal acquisition was made through a custom MatLab script (MatLab r2016b, MathWorks, USA) and the videos were synchronized with an output TTL to trigger the beginning and ending of each video. The aim of the ONEIROS system is to facilitate the recording of as many physiological parameters as possible in a more ecological manner for a prolonged period of time. The animals are freely moving and undisturbed by the weight of the implant (~11g for ONEIROS I and ~6g for ONEIROS II).

### *Minicam*

In one of the *Pogona vitticeps*, eye movement and eye closure were tracked using a custom made mini-camera system. This system used the NanEye miniature CMOS image sensor (AMS, USA). To get a proper illumination of the eyes, a custom-made structure was designed to align the mini-camera and an IR LED system (840nm). Eye movement videos were acquired by successive periods of 12h at 60Hz with a 249x250 pixels resolution in grey levels. This system allows for direct eye recordings with a negligible impact on the behaviour of the animals. Eye states were scored using a custom made MatLab script that evaluates eye opening based on the quantity of black (retina) in the picture (Figure 7).

### Electrode localisation

After brain slicing, Cresyl violet staining was done for all sacrificed animals. The paraffin was removed from each slice with two 4-min baths of methylcyclohexane, then two 4-min baths of 100% alcohol, followed by a 4-min bath of water. The Cresyl violet stain was performed by putting the slices successively into the following baths: 4 min Cresyl Violet acetate (1 g/L) (Sigma Aldrich, St. Louis, MO, USA), 2min 45sec alcohol 75%, 1min alcohol 90%, 30sec alcohol 100%, 15 s alcohol 100%, 5 s alcohol 100%, 2 min OTTIX (MM France, France). Stained slices were observed and capture using an Axioskop microscope (Axioskop 2 plus, Zeiss) and a colour video camera (ProgRes CF, Jenoptik). For the two *Stellagama stellio*, a CT scan post-



implantation was realised and realigned with the MRI and CT scan pre-implantation and prevent the sacrifice of our animals (for details see Libourel et al., 2018)

## Analysis

### *Behavioural scoring and electrophysiological extraction*

Wakefulness and sleep were scored based on the behaviour of the animal. An animal was considered sleeping if its head was laying on the floor and both of its eyes closed for at least a minute. Every other behaviour was considered wakefulness.

The electrophysiological parameters, namely, eye movements, muscle tone and heart rate and its variability were extracted, respectively, from the electro-oculogram (EOG), electrocardiogram (ECG) and electromyogram (EMG) using a custom MATLAB script. EMG signals were filtered using a High Pass filter at 10 Hz (removing signals below 10Hz) and a Band Stop filter between 45 and 55 Hz (removing signal between said limits to suppress electrical noise). Muscle tone was then extracted based on the on the absolute value of the Hilbert transform of the filtered EMG. EOG and ECG were filtered using a Low Pass filter at 10 Hz (removing signals above 10Hz). Peak detection (using “findpeaks” function, MATLAB) was used for EOG and ECG extraction with a peak height minimum determined by a threshold calculated for each animal independently and based on the mean of the absolute of the Hilbert transform of the signal plus one time the standard deviation for the eye movements or twice the standard deviation for the heart rate. The instantaneous heart rate variability was calculated as the standard deviation of the heart beat intervals. The minimum distance

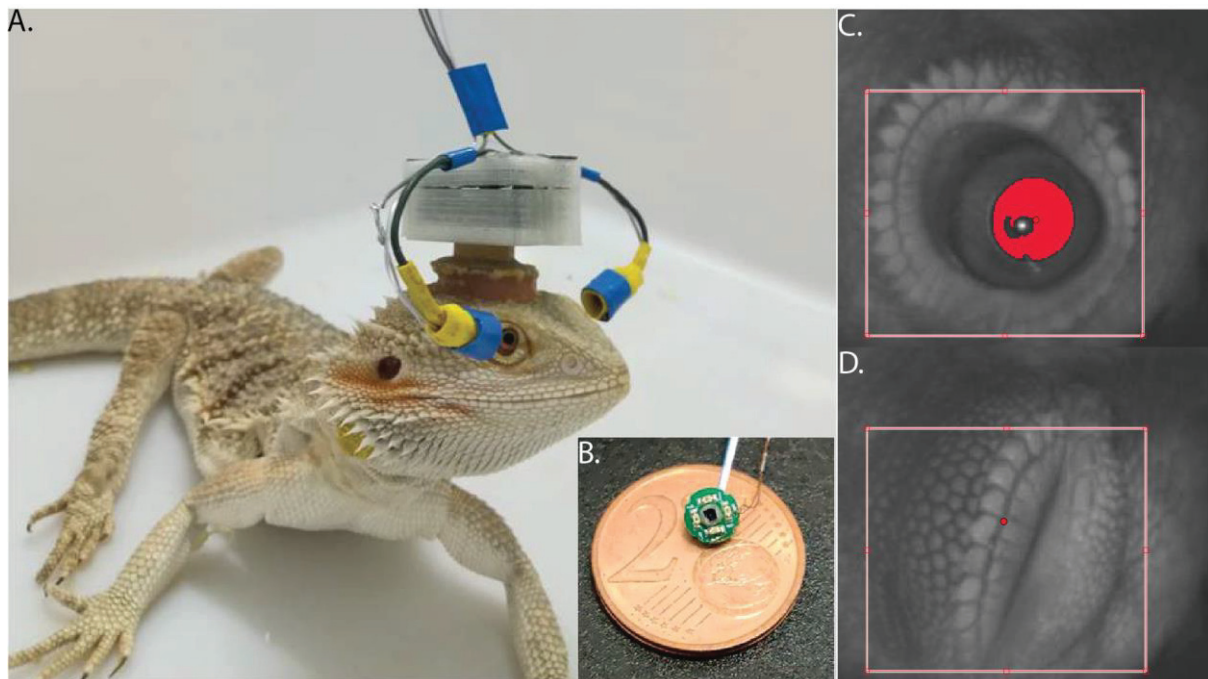


Figure 7 – Minicam prototype installed on a *Pogona vitticeps*. A. Picture of a *Pogona vitticeps* with the ONEIROS I headstage and the prototype of the MiniCam system. B. Picture of the minicam (black square) with the micro-IR LED system on a 2cts € coin. C&D print screen of the automatic eye state detection software looking at the retina (red ovoid shape) inside the region of interest (red square) with an example of the opened (C) and closed (D) eye.

between two peaks was set to be at least 0.1 second to avoid detection of different components of the same event.

#### *LFP Clustering*

We used the same methodology described in previous papers (Libourel et al., 2018; Shein-Idelson et al., 2016). From the baseline experiments, between 9 PM and 2 AM, the signal of the chosen electrode was whitened with an autoregressive algorithm. A multitaper power spectrum between 0.5 and 30 Hz was computed for each 3-s epoch (windows 3 s, bandwidth 1 Hz, 5 tapers) ([Chronux](#)). Each power spectrum was normalized by the mean power spectrum. A correlation matrix of these power spectra was calculated. Then, a hierarchical clustering with two clusters was realized based on a Euclidian distance of the correlation and using a Ward linkage. A mean normalized power spectrum per animal was then calculated for each cluster.

#### *High voltage waves extraction*

The HvWs were extracted using a custom Matlab script. Peak detection was used with a peak high minimum determined by a threshold calculated for each animal independently and based on the 95<sup>th</sup> percentile of the raw LFP data. The channel used for HvW extraction was selected visually based on the best ratio between peak height and signal noise. Detected HvW were checked to confirm that transient artifacts mainly during active wake were not included.

#### *Signal Power analysis*

The signal power for each band frequency was extracted by filtering the raw data in the limits defined earlier. The filtered data was smoothed by calculating the root-mean-square envelope with a 0.5 second window and normalised by the instantaneous raw power (whole band smoothed with the same method). The mean of the normalised envelope was calculated per bout of 1 minute and a moving mean with a moving window of 10 minutes was applied to smooth the data.

#### *Respiration recordings*

In the *Broadleysaurus major*, the respiratory frequencies were extracted using a custom MatLab script. This script uses the `opticalflowLK` function that estimates the direction and speed of object motion from previous video frame to the current one using Lucas-Kanade algorithm in a region of interest. Noise threshold was set at 1/10000. The region of interest (ROI) was determined individually for each animal to optimize the movement detection associated with respiration. The ROI was set on the lateral part of the torso behind the front limb.

## Study 1 – *Agamidae* sleep

The Agamidae encompass around 500 species (*The Reptile Database*, Uetz). They appeared some 98 MYA ago and are now found in Africa, Asia, Australia, and the southern part of Europe. They are divided in 6 subfamilies and contain the two species of interest in this study the bearded dragon, *Pogona vitticeps*, and the starred agama, *Stellagama stellio*. These two species shared a similar environment. The *Pogona vitticeps* lives in the rocky desert of Australia when the *Stellagama stellio* is found in the rocky parts of Greece, Israel, and western Asia. The aim of this study was to compare the behavioural & physiological components (e.g., sleep quantities and phenotype, brain patterns and evolution, ...) of sleep in the bearded dragon to that of *Stellagama stellio* (Fig. 8). By looking at two, evolutionary speaking, close species (83 MYA apart), we would like to determine whether the unusual sleep phenotype of the bearded dragon (i.e., its cyclic nature, the stereotypic brain oscillations or the occurrence of eye movement while sleeping) is a common phenotype in Agamidae or whether it is specific to the bearded dragon. In addition, we will look at other mammalian sleep features (muscle tone, heart rate, breathing rate, twitches, ...) in both species, to give a better description of this lizard's sleep. By doing so we hope to provide more clues into the inference of lizard sleep states.

The first part of this study aims to describe and compare sleep behaviour and physiology (e.g., brain activity, muscle tone, eye movements...) in the two species mentioned. The second parts take a closer look at the uncommon behaviour and physiology of the bearded dragon by looking at unilateral eye closure.

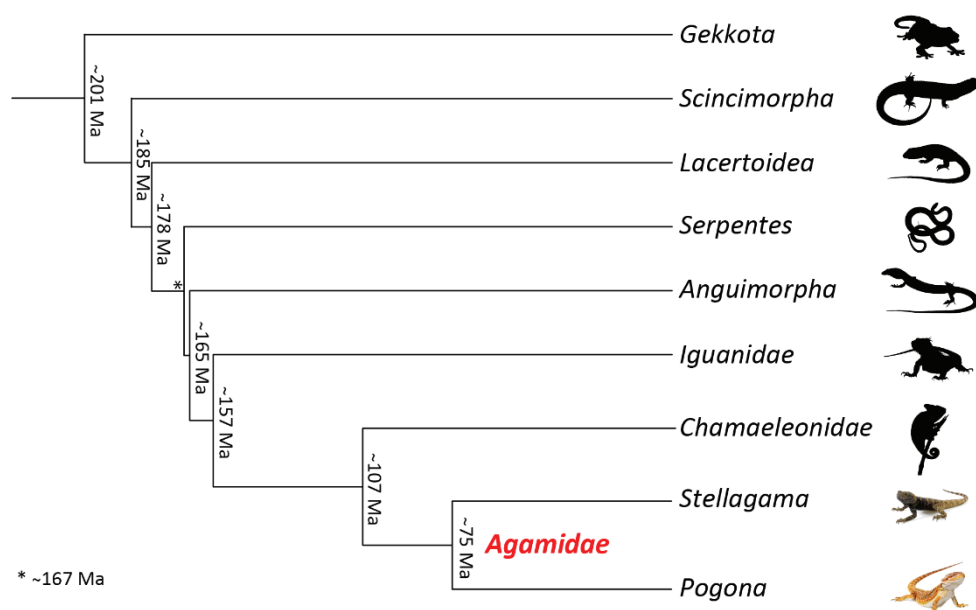


Figure 8 – Schematic representation of the Squamata phylogenetic tree represented by the major groups of species with a focus on the Agamidae group. The silhouettes are up to scale. Ma = Million years

## Part I – *Pogona vitticeps* & *Stellagama stellio* sleep

### Behavioural sleep in *Stellagama stellio* & *Pogona vitticeps*

Behavioural sleep has been identified in every individual based on the head elevation and the eye status (Huntley, 1987). The animals are almost exclusively active during the light period of their day, which is coherent with observations in the wild. The awakening onset of the animals was approximatively 38 min (+/- 40 min) after the light onset for the bearded dragons and 19 min (+/- 36 min) for the starred agama. When time approaches the dark period, bearded dragons as well as the *Stellagama stellio* assume their rest position with the head laying down but with their eyes open. Behavioural sleep onset (when animals in sleeping position closed both eyes for more than 2 minutes) was at 22 min (+/- 37 min) before the light offset for the *Pogona vitticeps* and 32 min (+/- 45 min) for the starred agama. Behavioural sleep in these species is a reinforced unique period that continues through the night with a duration of 12h 16 min (+/- 58 min) for *Pogona vitticeps* and 12h 50 min (+/- 45 min) for *Stellagama stellio*. Like the argentine tegu (Libourel et al., 2018), Behavioural sleep was also correlated with a diminution of the number of eye movements as well as muscle tone and heart rate, when stable recordings were available, in both species (Fig. 9). During this period, rare limb and tail movements were recorded.

### Identification of two distinct brain activities during behavioural sleep

Using unsupervised hierarchical clustering on the brain activity recorded in the DVR during the behavioural sleep of the animals, we replicated the data found in the *Pogona vitticeps* in previous studies and we found two distinct brain activity types in *Stellagama stellio* as well. Our recordings in the bearded dragons showed, as described previously, one electroencephalographic state with high LFP power in the lower frequencies (i.e., 0.5-4 Hz) and a broader one with high LFP power in frequencies above 10Hz. The two brain activity types identified in the starred agama are similar the two states of the bearded dragon with one “slow” state with high LFP power in the lower frequencies (0.5-6Hz) and a broader, more desynchronised state, with high LFP power in frequencies above 10 Hz (Fig. 10).

By separating the two main spectral contents of the LFP during behavioural sleep, the clustering helped determine the best power ratio to automatically detect and score the two states. We therefore used the [10-30 Hz] power ratio to distinguish and score the bearded dragon and the *Stellagama stellio* electrophysiological sleep states.

More than just revealing two electrophysiological sleep states in the starred agama, raw data showed that they were similar in term of spectral content to those reported in the bearded dragon. The first state (S1) is bereft of overall brain activity. It shows low activity in most band powers except for the delta activity (0.5-4 Hz) and displays isolated high amplitude HvW (Fig. 11). Those HvW are not as similar as the rest of the brain activity compared to the ones recorded in the *Pogona vitticeps*. They are mostly composed of a positive deflection in our recordings whereas the bearded dragon HvW are mostly negative (Fig. S1 & S2). The second state (S2) on the other hand is more packed.

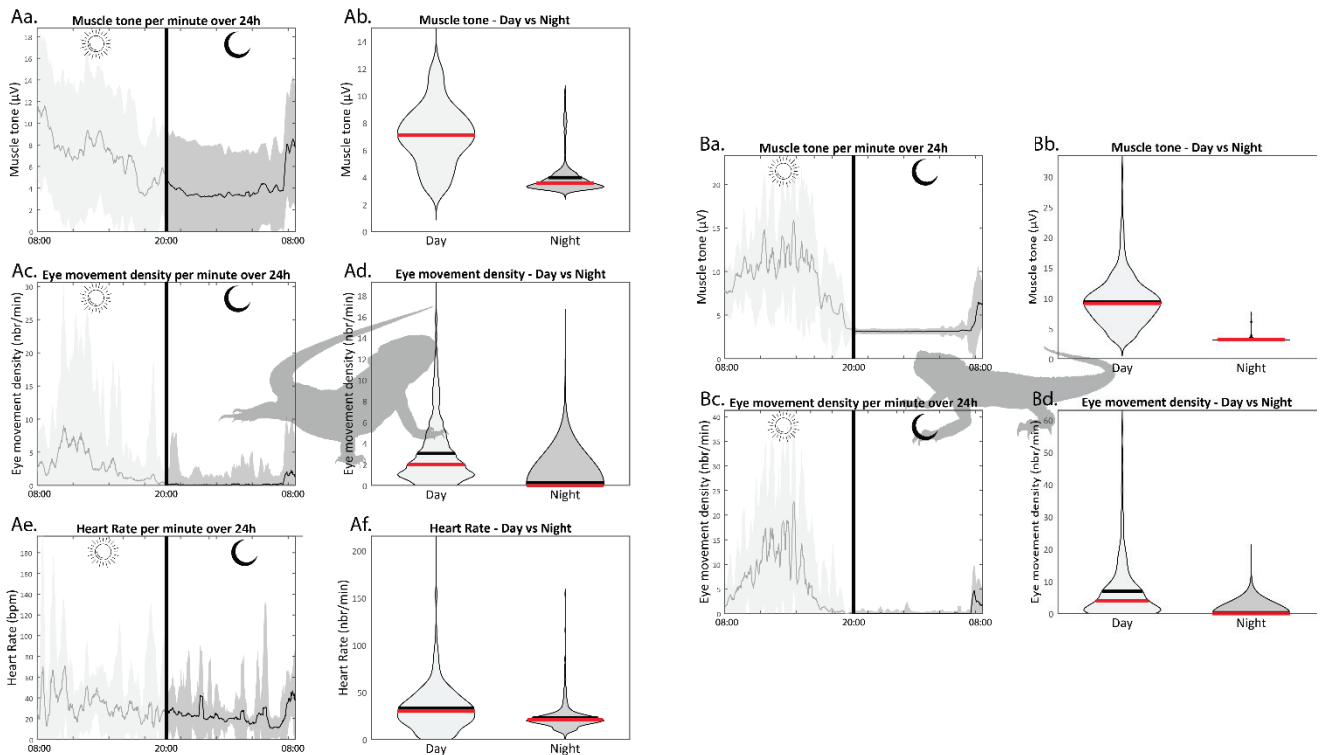


Figure 9 – Physiology evolution over 24h in the bearded dragons and starred agama. A. Physiology of the *Pogona vitticeps* (4 individuals, n=11). Smoothed evolution per minute over 24h of respectively the muscle tone (a), the eye movements density (c) and the heart rate (e). Violin plot of the distribution during the day (blue) versus the night (orange) of the muscle tone (b), the eye movements density (d) and the heart rate (f) B. Idem as A but for the *Stellagama stellio* (2 individuals, n=7), except for the heart rate e&f that was not recorded. The red bars indicate the median value and the black bars the mean.

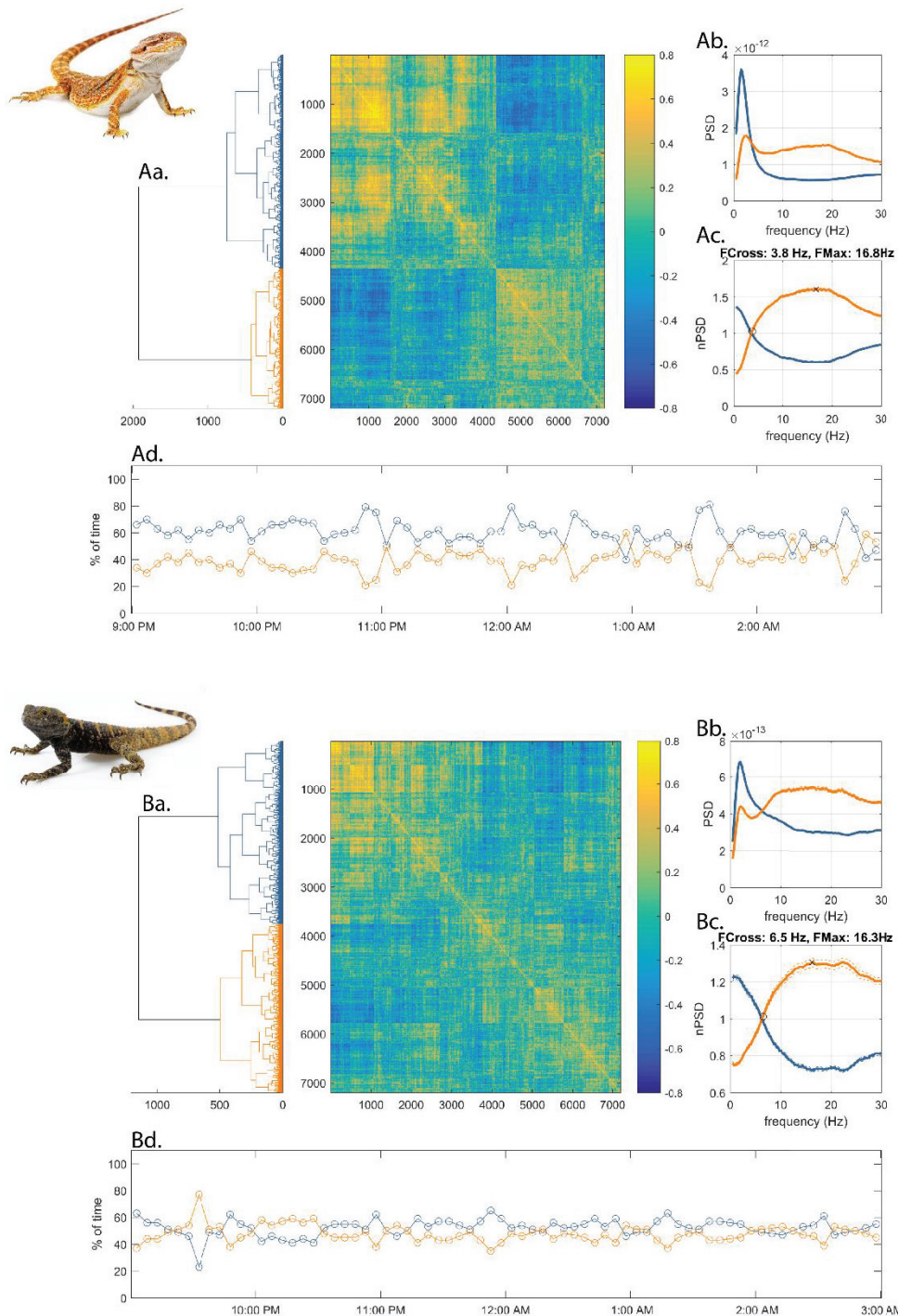


Figure 10 – Clustering analysis of *Pogona vitticeps* (A) and *Stellagama stellio* (B) brain activity during sleep behaviour. With a. unsupervised hierarchical clustering, b. Power spectrum of the two electrophysiological states between 0 & 30 Hz, c. Normalised PS of the two states, and d. Relative quantities of each state per 5 min over 6 hours (21:00 to 03:00).

It shows broad band desynchronised brain activity similar to recordings during the waking period.

In the *Pogona vitticeps* sleep, one of the most puzzling particularities was its overall quantity. With almost 45.7% (+/- 9%) of the S1 and 54.3% (+/- 9%) of S2, they display a unique ratio in sleep states quantities in the animal kingdom (Libourel & Barrillot, 2020) that roughly translate to 5.5h spend in S1 and 6.5h spend in S2. The starred agama also shows unprecedented sleep ratio with S1 brain activity representing for 62.6% (+/-3%) or roughly 8h of the sleeping behaviour and S2 counting for 37.4% (+/- 3%) or roughly 4.8h (Fig. 13). Even if those quantities are not as unusual as the bearded dragon ones, they still represent a notable difference in the quantity of REMS like activity compared to the ones recorded in mammals.

#### Physiological characterisation of the two sleep states

We looked at the muscle tone and the eye movements those 2 parameters being defining points of the division between SWS and REMS in mammals and birds. The muscle tone was significantly higher during the waking states compared to the sleeping ones, in both species. However, no notable difference was recorded between the two sleep states neither in *Pogona vitticeps* nor in *Stellagama stellio*. In the bearded dragon, the heart rate also decreases during behavioural sleep compared to wake similar to what was recorded in the Argentine tegu (Libourel et al., 2018) but no differences were present between S1 and S2 (Fig. 12).

Regarding the eye movements, in the bearded dragon it was reported (Libourel et al., 2018; Shein-Idelson et al., 2016) that they occur preferentially, but not only, during S2, and during the first part of the state. Here we also reported very few eye movements during S1 in both species with density of 0.473 eye movements per minute for *Pogona vitticeps* and only 0.143 eye movements per minute for *Stellagama stellio*. Whereas eye movements in S2 are more numerous in both species with a density of 0.690 for the bearded dragons and 0.482 for the starred agamas. This is also visible in the percentage of sleep events displaying at least one ocular movement. In *Pogona vitticeps*, only 21% of S1 episodes contain at least one eye movement versus 38% for S2. In *Stellagama stellio*, the difference is even more striking with only 7% of S1 episodes displaying at least one eye movement versus 26% for S2. Lastly, no visible differences were reported in the amplitude of the eye movements during any of the vigilant states in both species.

In the *Pogona vitticeps*, Brain temperature recordings showed that no significant change occurred when transitioning from S1 to S2 (Fig.14). No significant change happened during the night expect for a slow and steady decrease of the temperature reflecting the decline of temperature across the night (See Supplementary I).

As for twitches, rare movements of the limbs, tail or head were recorded in both our species. Those movements were rarer in *Stellagama stellio* than they were in *Pogona vitticeps*. They happened 26 (+/-2) times during the starred agama's sleep, or once every

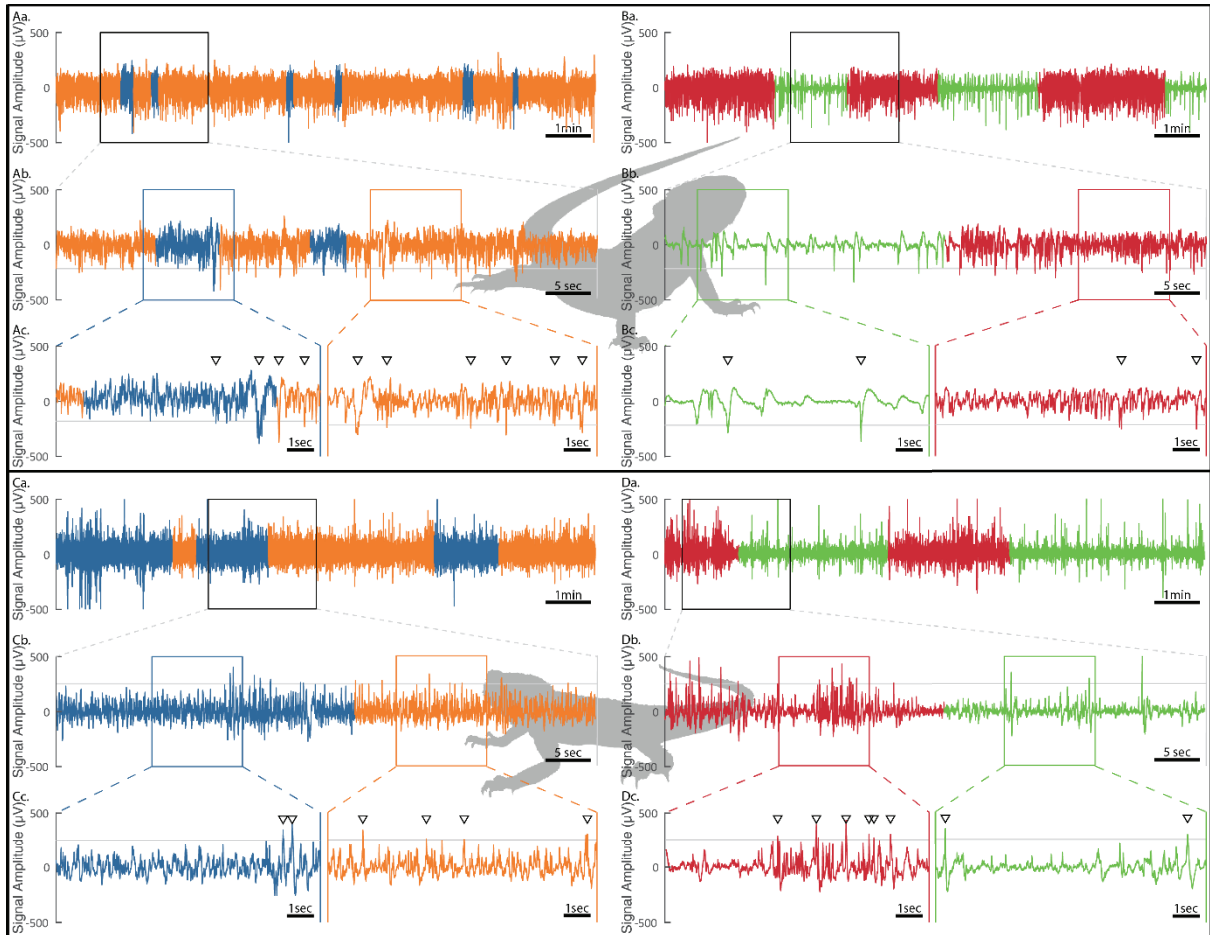


Figure 11 – Raw DVR LFP data of the *Pogona vitticeps* wake states (A), and sleep states (B) & of the *Stellagama stellio* wake states (C) and sleep states (D) for (a) 5 min, (b) 1 min, (c&d) 10 seconds. Blue = Active Wake, Orange = Quiet Wake, Green = Sleep 1, and Red = Sleep 2. The arrowheads show detected HvW and the grey line in A, B, C, & D b, c, & d is the threshold used to detect them (i.e. 2 times the 95% quantile of 24h of data).



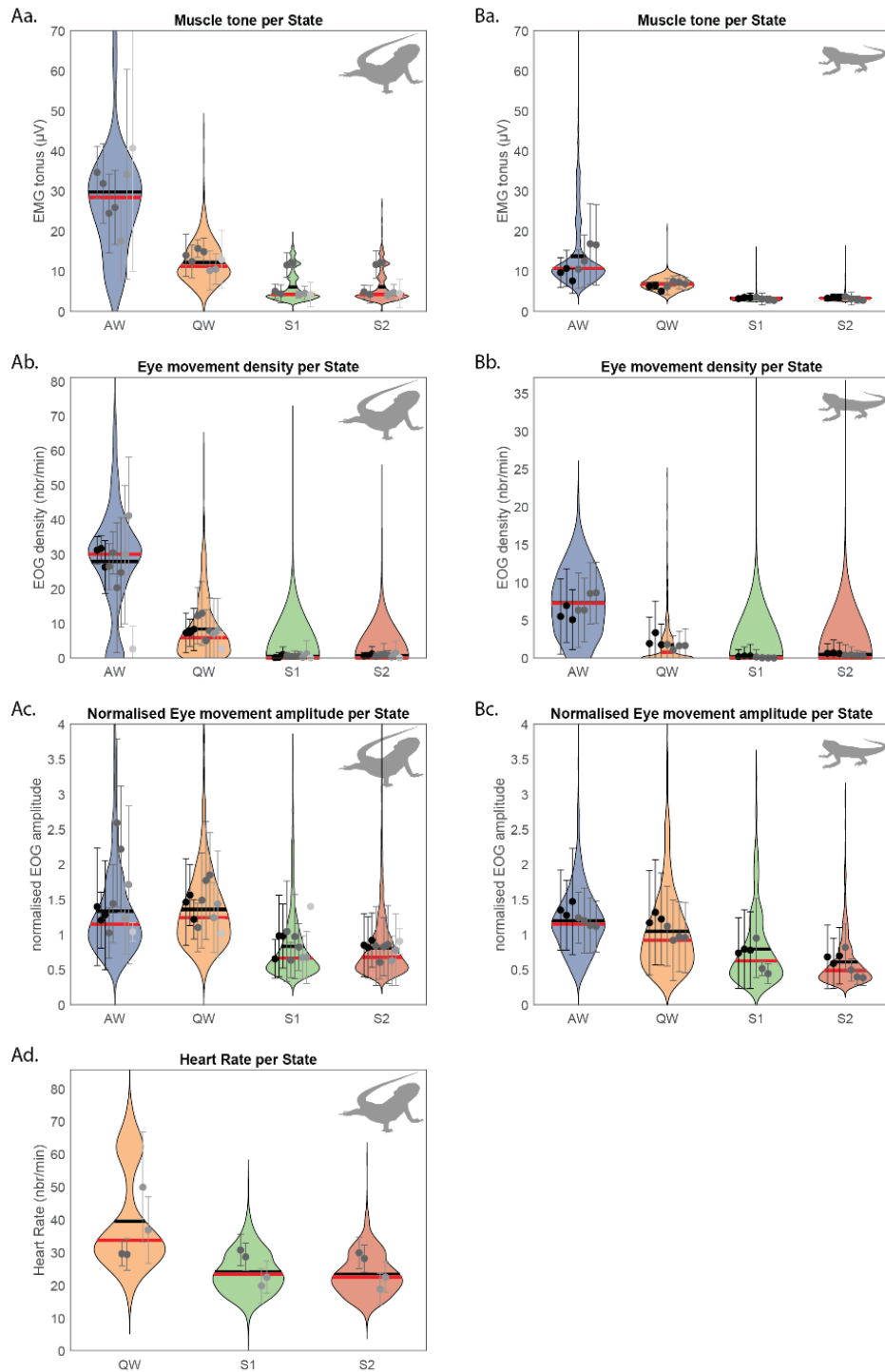


Figure 12 – Physiology of the vigilant states of *Pogona vitticeps* (A) and *Stellagama stellio* (B, 2 individuals,  $n=7$ ). a. Violin plot representation of the distribution of the muscle tone in  $\mu\text{V}$  for every vigilant state (Active Wake = AW in blue, Quiet Wake = QW in orange, Sleep 1= S1 in green, and Sleep 2= S2 in red, Aa. 3 individuals,  $n=7$ ) b. Violin plot representation of the eye movement density distribution for every vigilant state (Ab. 4 individuals,  $n=10$ ). c. Violin plot representation of the normalised eye movement amplitude distribution for every vigilant state (Ac. 4 individuals,  $n=10$ ). d. Violin plot representation of the heart rate distribution for every vigilant state (only available for the *Pogona vitticeps*, Ad. 2 individuals,  $n=4$ ).

16 (+/- 1) cycles, in the starred agama and 128 (+/-9) times during the bearded dragon's sleep, or once every 2.8 (+/- 0.2) cycles, in the bearded dragons. They occurred in both states and were slightly more present during S1 in both species (62.4% +/-32 for the *Stellagama stellio* and 55% +/-19 for the *Pogona vitticeps*) (Fig. 15).

#### High voltage waves & sleep

Hippocampal Sharp waves ripple complexes, are in mammals (*i.e.* rodents) is associated with “off-line” brain states and in particular NREMS (Buzsáki, 2015). The LFPs of both species are marked by the occurrence of High voltage Waves (HvW). For *Pogona vitticeps*, the HvW were clearly detected in S1 but were also detected in every vigilant state. They have a mean amplitude of 308  $\mu\text{V}$  (+/- 153 $\mu\text{V}$ ) with a mean width of 111 msec (+/- 0.174 ms). They are expressed throughout the night with no visible prevalence for the beginning or the middle of the night like as it was described in the Argentine tegu. For the *Stellagama stellio*, HvW were also detected in all the vigilant states with a mean amplitude of 211  $\mu\text{V}$  (+/- 106 $\mu\text{V}$ ) and a mean width of 81 msec (+/- 155 msec). The number of HvW per minute was notably higher during the Active Wake (AW) and Quiet Wake (QW) compared to S1 but not S2 (respectively 12.1+/-3.4 pks/min, 10.0+/-3.2 pks/min, 3.85+/-2.9 pks/min & 5.21+/-3.6 pks/min). No notable differences were recorded for the amplitude or the width of the HvW between the different vigilant states for both species (Fig. 16).

#### Sleep states and their rhythmicity

One key feature of the *Pogona vitticeps* sleep is the regularity of the alternance between electrophysiological sub states. It has been shown, and we replicated here, that the two sleep states of the bearded dragon alternate regularly throughout their sleeping period with a period of 80-90 sec at 27°C (Libourel et al., 2018; Shein-Idelson et al., 2016). In our study (25°C), we report that one sleep cycle lasts 122 seconds (+/- 54) with 46% spend in S1 and 54% in S2. This clocklike regularity is also present in *Stellagama stellio* with a period of 113 (+/- 40) seconds with 63% spent in S1 and 37% spent in S2.

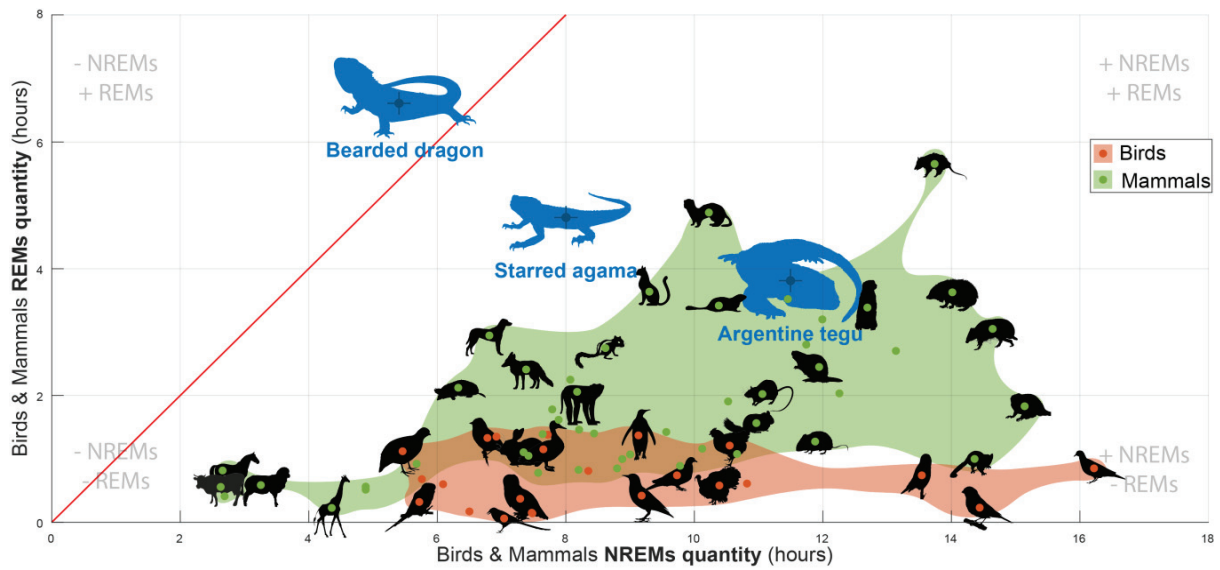


Figure 13 – Updated figure from Libourel & Barrillot, 2020. Ratio of mammalian & avian REMS over NREMS compared to their putative squamates equivalents over 24 hours. The relative quantities of the two types of sleep for squamates are calculated based on the assumption that S1 is an equivalent of SWS and S2 the equivalent of REMS. The relative quantities were calculated based on the results found in the literature and our experiments (Capellini et al., 2008; Roth et al., 2006, Libourel et al., 2018). The corners of the graph represent the minimal (-) and maximal (+) quantities of NREMS and REMS. For example, the top right corner represents the maximal quantities of both NREMS and REMS (+NREMS/+REMS).

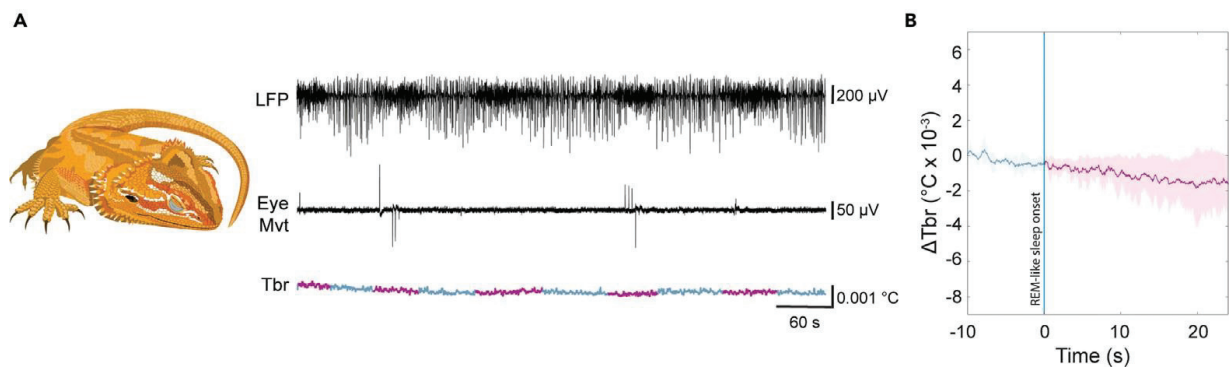


Figure 14 – A. 10-min local field potential (LFP) recording from the dorsal ventricular ridge (DVR) of a sleeping bearded dragon showing state-related changes in brain activity. The brain states are colour coded (S1, cyan; S2, magenta) in the bottom brain temperature (Tbr) recording. The electrooculogram (Eye Mvt) shows the association between eye movements and brain state. B. Tbr recorded from the DVR of sleeping bearded dragons 10 s before and 30 s after the transition (vertical blue bar) between S1 and S2. Tbr values (mean $\pm$  standard deviation; see Supplementary I for individual data) are expressed relative to Tbr 10 s before the transition. Bearded dragon illustration by Damond Kyllö. Extracted from Ungurean et al., 2020 (see Supplementary I).

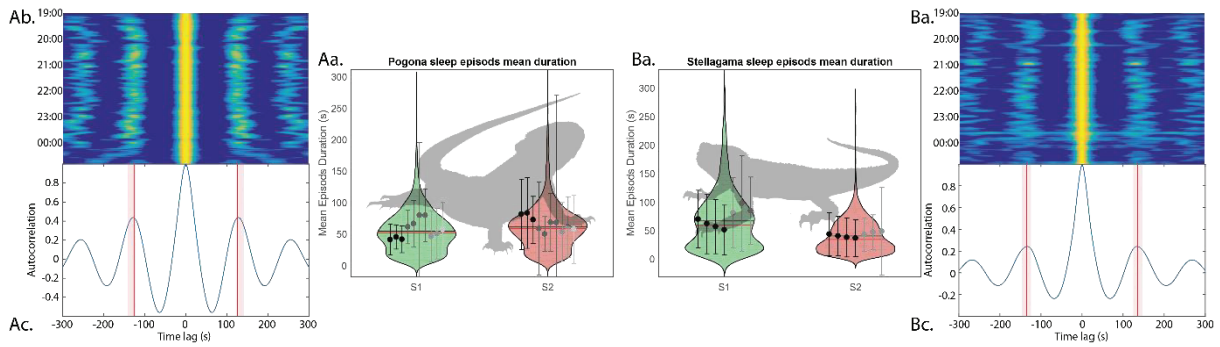
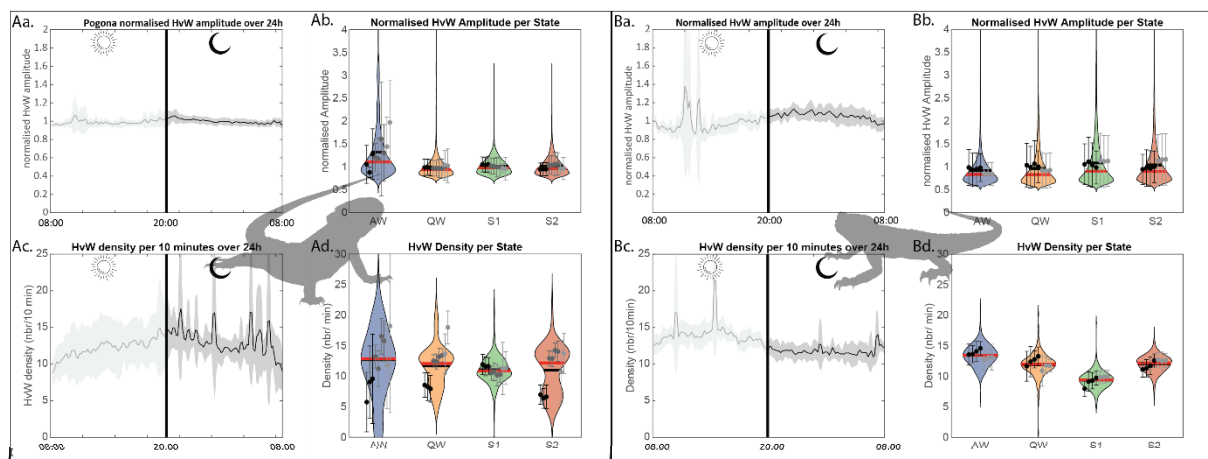


Figure 15 –Sleep pattern and rhythmicity in the *Pogona vitticeps* (A) and the *Stellagama stellio* (B). a. Violin plot representation of S1 and S2 episodes duration distribution. Grey dots with error bar are mean individual recordings +/- std. Each shade is one individual with its replication (*Pogona vitticeps* n=4 with respectively 3, 4, 2, & 1 replications & *Stellagama stellio* n=2 with respectively 4 and 3 replications). b. Autocorrelation map of the DVR  $\beta$  signal over 12h of night of one representative individual. c. Mean autocorrelation over the 12 hours. Red bar is the median peak and the shade the IQR.



24h distribution of the mean HvW density per minute (light purple – Day, dark purple – Night) b. Violin plot representation of the distribution of the HvW density for every vigilant state (Active Wake = AW in blue, Quiet Wake = QW in orange, Sleep 1= S1 in green, and Sleep 2= S2 in red). c. Graphical representation of the 24h distribution of the mean HvW amplitude per minute. d. Violin plot representation of the distribution of the HvW amplitude for every vigilant state. Mean HvW wave form available in Figure S1 & S2.

## Part 2 – *Pogona vitticeps* & Brain Asymmetry

Our observations allowed to describe a new feature of bearded dragon sleep behaviour, unilateral eye closure (UEC) during wakefulness. We added micro-cameras to one of our animals allowing the characterisation and quantification of UEC and the brain activity associated with it.

### Behavioural characterisation of unilateral eye closure

Based on the automatic detection of each eye opening, we were able to automatically score the eye states of our animal (Fig. 7). UEC episode duration was the same independently of the eye and was around 16 seconds long (IQR [10-31]). The automatic scoring over 24h revealed that UEC of either eye increases at light onset and plateaus after 2h at 28% (IQR [27-38]) of the time for the right eye and 8% (IQR [7-9]) for the left eye until 2h before light offset were UEC starts to diminish (Fig. 17). Interestingly, the animal spent the majority of the time with both eyes open only during the first part of the day the reaching a plateau at 42% (IQR [35-51]) and this proportion rapidly decreased after midday in favour of a both-eyes-closed behaviour that increase rapidly.

### Brain asynchrony during UEC

During UEC episodes, LFP recordings showed that the contralateral hemisphere to the closed eye expressed a similar pattern to what was recorded during S1 as soon as the animal closes its eye. HvW superimposed on the decreasing broad awake activity (Fig. 11). The PS analysis show that the frequency content is highly similar between the activity of the brain hemisphere when both eyes are open and when the ipsilateral eye is closed and between the hemisphere activity when both eyes are closed and when the contralateral eye is closed (Fig. 18 & 19).

## Main results

This study highlights five major points:

- The rhythmic alternance between two cerebral states exists in another *Agamidae* species, the *Stellagama stellio*.
- S2 seems to show a prevalence of eye movements in the two species.
- The HvW are not specific to S1 but occurs in all vigilant states, including wake.
- No major change occurs in the muscle tone, brain temperature and heart rate between S1 and S2. Twitches occurred in both states.
- The *Pogona vitticeps* displays unilateral brain asynchrony associated with UEC during its waking period.

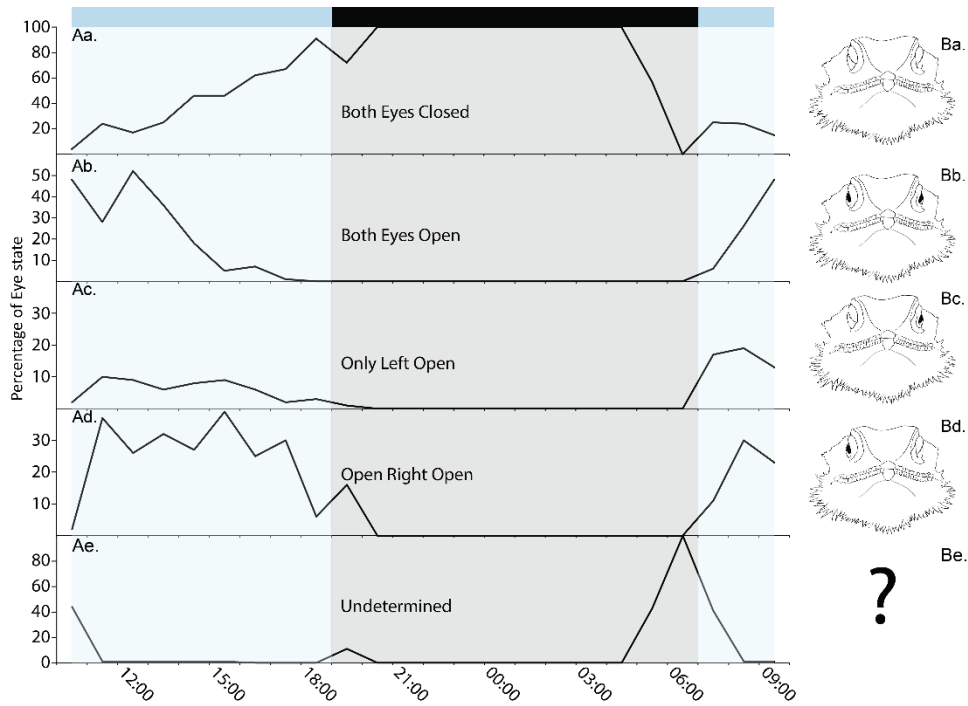


Figure 17 – Representation of opening/closure of the eyes in *Pogona vitticeps* for 24h. A. Mean percentage of each state of eye opening (a. both eyes closed, b. both eyes opened, c. only left eye opened, d. only right eye opened, e. undetermined) for every hour over 24h. Light blue zone - Lights on, Dark grey zone - Lights off. B. Schematisation of the different eye states of the animals (a., b., c., d., e. same as A).

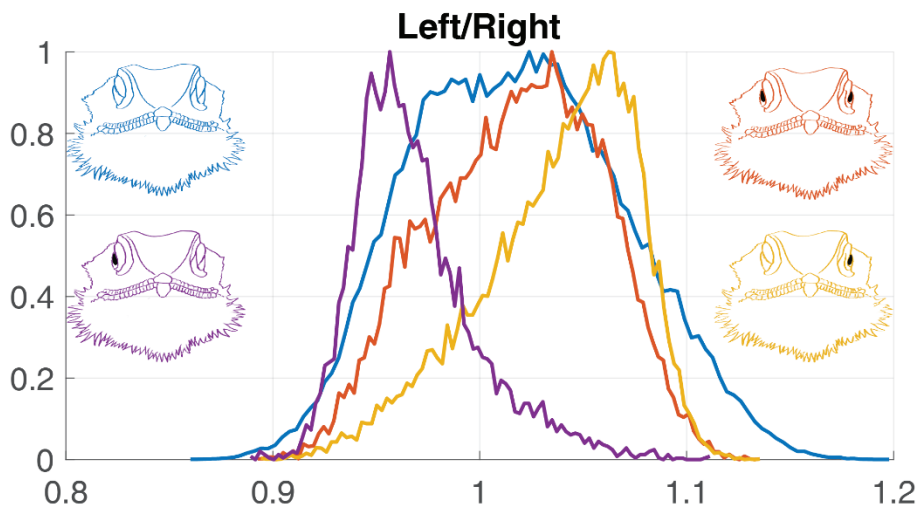


Figure 18 – Ratio of the beta power of the left hemisphere over the beta power of the right hemisphere when the animal has both of its eyes closed (blue), both of its eyes open (orange), only the left eye open (yellow) or only the right eye open (purple).

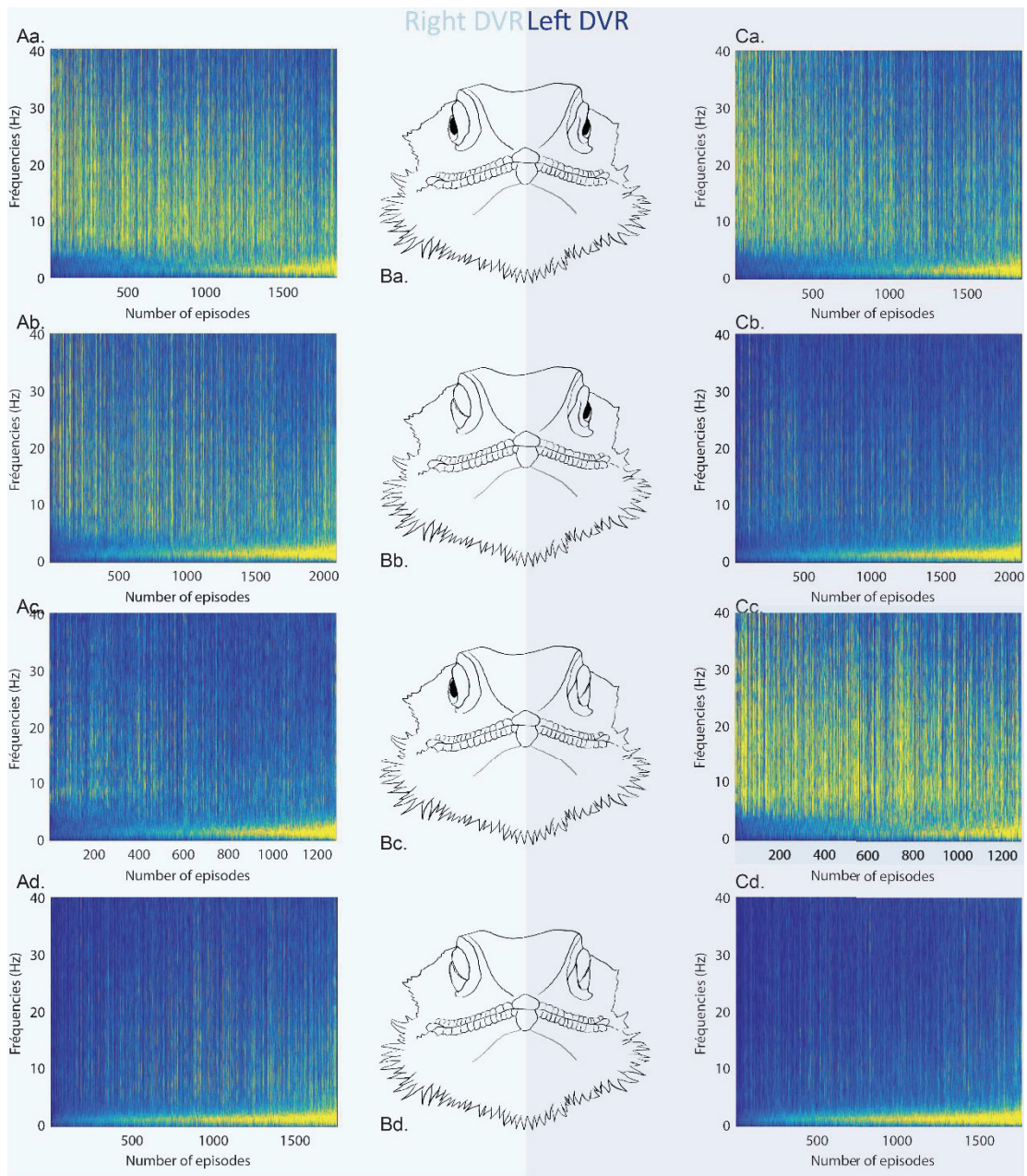


Figure 19 – Spectral representation of the brain activity of *Pogona vitticeps* relative to the eye-opening status. A. Mean power spectrum of the brain activity in the Right DVR of each episode of the different eye-opening states (a. both eyes close, b. both eyes opened, c. only left eye opened, d. only right eye opened) sorted based on the quantity of slow wave (0.5-5hz). B. Schematics of the different eye-opening status (a., b., c., & d. idem A). C. Mean power spectrum of the brain activity in the Left DVR of each episode of the different eye-opening states (a., b., c., & d. idem A) sorted based on the quantity of slow wave (0.5-5hz).

## Study 2 – Scincoidae sleep

In this study, we explored sleep in another subgroup of squamates, the *Scincimorpha*. This group of lizards appeared almost 155 MYA ago and count more than 1500 distinct species. They are characterised by a stout cylindrical body and short legs. Our species of interest, the Sudan plated lizard or *Broadleysaurus major*, is easily identified by its heavily armoured appearance with thick keeled square scales. They are primarily found in the Eastern Africa and prefer dry rocky environments. The *Scincimorpha* branch is almost at the root of the Squamata tree and by studying this new species using the same technique used in the previous studies, we want to gain some information on their sleep behaviour and determine what features of the lizard sleep phenotype is ancestral or evolved independently (Fig. 20).

Sleep in the Sudan plated lizard...

Based on the same parameters used in the previous studies, sleep behaviour has been identified in the Sudan plated lizard. Like their *Agamidae* counterparts, *Broadleysaurus major* sleep behaviour is notable based on the position of their head, laid down, the states of their eyes, closed, and the position of their limbs, relaxed and spread outward. Their sleep behaviour mostly happened during the dark phase and their sleep and wake onset were respectively 20:15 (+/- 26 minutes) and 08:02 (+/- 4 minutes).

... and its physiological parameters.

Like previously shown in the *Agamidae* (cf Study 1 – Part 1), sleep behaviour in the Sudan plated lizard was characterised by a global diminution of physiological parameters with notably less eye movements (0.67+/-1.3 eye movements per minute during the day versus 0.036+/-0.24 eye movements per minute during the night) and a slight diminution of the

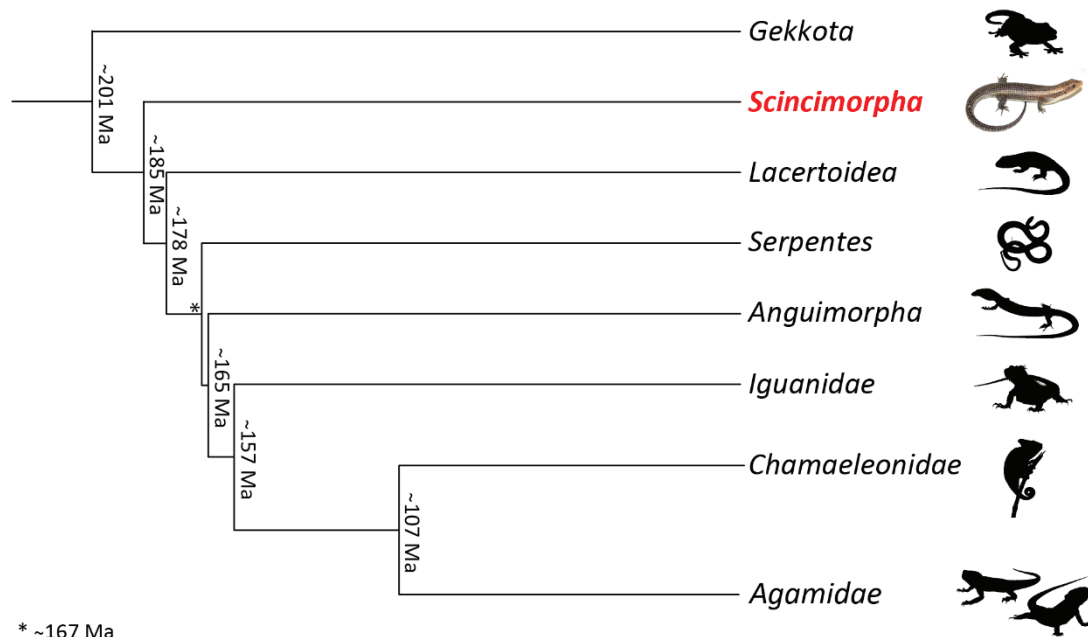


Figure 20 – Squamata phylogenetic tree highlighting the position of *Broadleysaurus major*. The silhouettes are not to scale. Ma = Million years.



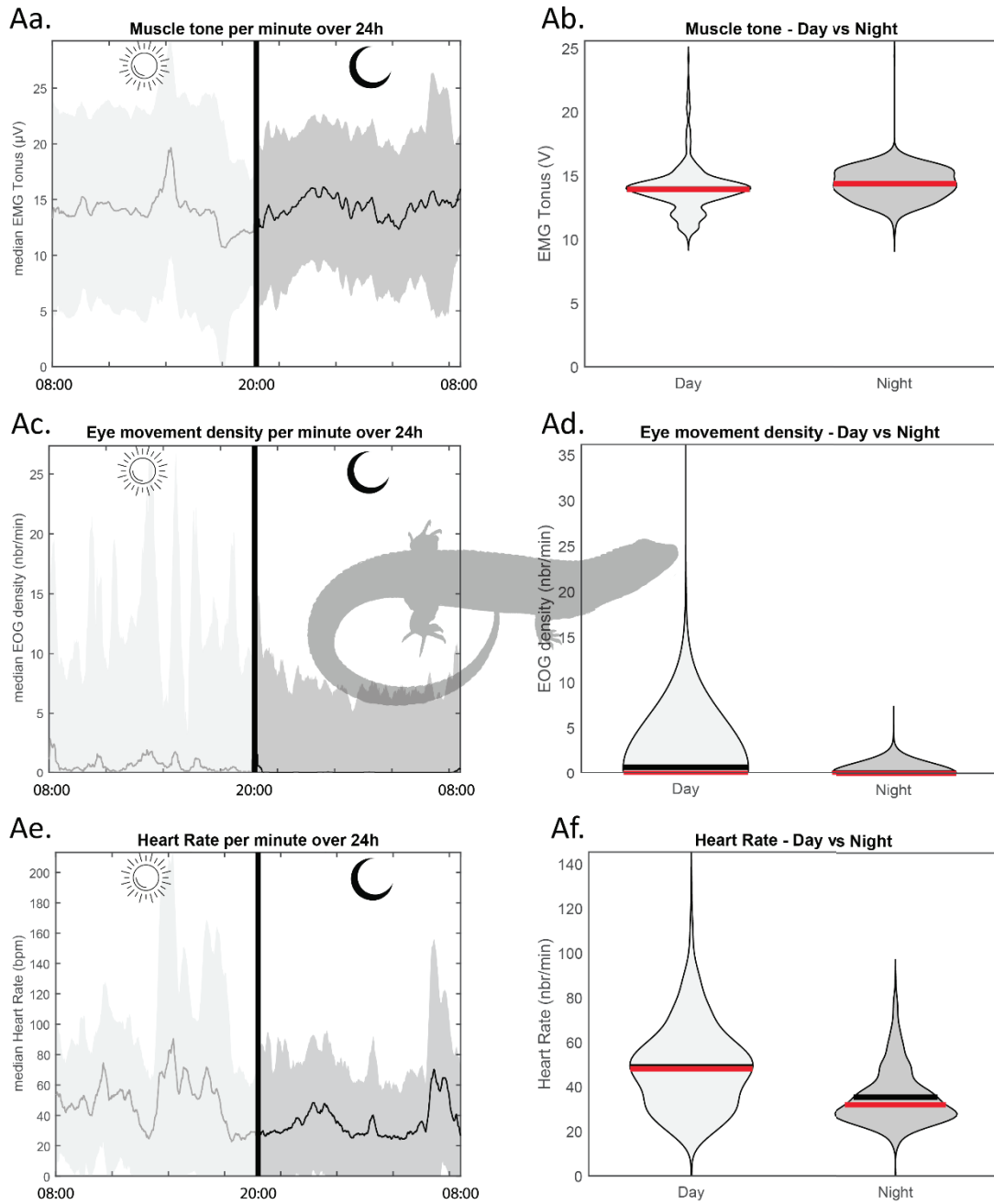


Figure 21 – Physiology evolution over 24h in the Sudan plated lizard (3 individuals, n=11). Smoothed evolution per minute over 24h of respectively the muscle tone (a), the eye movements density (c) and the heart rate (e). Violin plot of the distribution during the day (blue) versus the night (light orange) of the muscle tone (b), the eye movements density (d) and the heart rate (f). Red bar is the median value and black bar is the mean value.

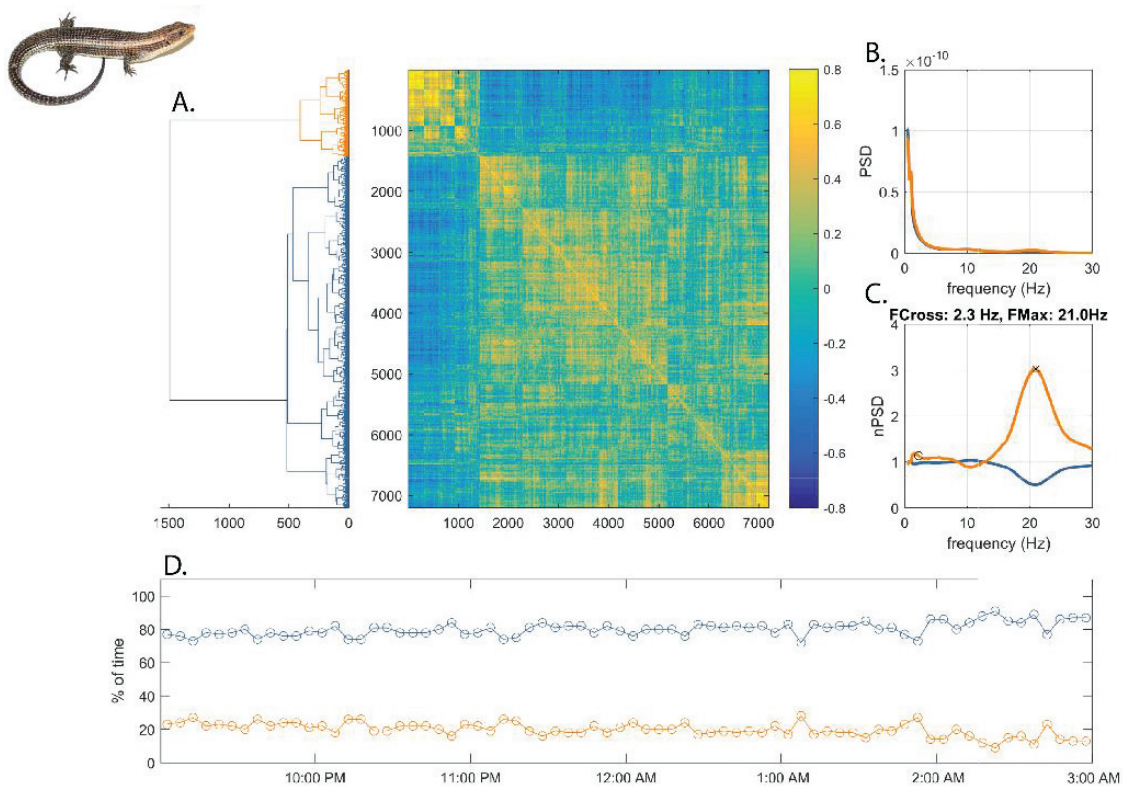


Figure 22 – Clustering analysis of *Brodleysaurus major* sleep behaviour brain activity. With a. unsupervised hierarchical clustering, b. Power spectrum of the two electrophysiological states between 0 & 30 Hz, c. Normalised PS of the two states, and d. Relative quantities of each state per 5 min over 6 hours (21:00 to 03:00).

heart rate (42 $\pm$ 18 BPM during the day versus 39 $\pm$ 15 BPM during the night). The muscle tone seems to show no physiologically significant differences between wake and sleep (13.6 $\pm$ 1.6  $\mu$ V during the wake period versus 14.5 $\pm$ 1.3 $\mu$ V during the sleep period) (Fig.21).

Are there two sleep states in *Broadleysaurus major*?

Based on the results of the clustering method used previously, two oscillatory patterns exist in the DVR during the sleeping period of the Sudan plated lizard. One state with low power in all the bands representing 80% of the considered period (between 9PM and 3AM) and a second one with a similar low frequency characterised by an oscillation at 20 $\pm$ 1,5 Hz (Fig. 22).

The analysis of the raw data showed that 20 Hz oscillation was happening quite regularly every 25 sec  $\pm$  22 sec in less than a second-long burst (0.69 sec  $\pm$  0.15sec) of beta activity. Those burst of activity were also identified during the waking period at a smaller interval, 11 sec  $\pm$  15 sec (Fig. 23). Looking at the evolution of the other physiological parameters during those bursts of activity, the presence of respiration was highly correlated with the occurrence of the bursts. Every burst of 20Hz activity happened during a respiration (Fig. 24).

No other clear frequency pattern was found by computing a clustering with three expected clusters and the overall power of all the frequency bands tends to diminish between day and night (Fig. S4).

A rhythm throughout the night?

The DVR activity (except for the 20 Hz oscillation associated with the respiration) does not show any major difference in the LFP, like reported in the two *Agamidae*. However, a periodicity in the signal amplitude seems to exist in all frequency bands (Fig. 25A). This regular fluctuation was more prominent in one animal but still present in all lizards recorded. Overall, a 0.01Hz  $\pm$  0.002Hz (88.5 sec periods  $\pm$  11 sec) was visible throughout the nights in all animals (Fig. 25).

The physiological evolution during the sleeping period

The analysis of the EMG & ECG did not show any sign of a rhythmicity throughout the sleeping periods of the animals as the LFP does. However, while this needs to be confirmed with additional processing, the eye movement of the animals seems to appear preferentially during the “upstate” of the LFP oscillation (Fig. 25).

## Main Results

- The Sudan plated lizard present a 20 Hz oscillation in the DVR associated with its respiratory rate.
- No major change seems to occur in the spectral content of the DVR LFP during the sleep wake cycle.
- A 0.01Hz oscillation is however visible in all the frequency bands during the sleeping behaviour and associated with eye movements during the upstate.
- No other physiological changes seem to be associated with this power change.
- No clear signs of HvW were found in any vigilant state of the Sudan plated lizard DVR LFP.

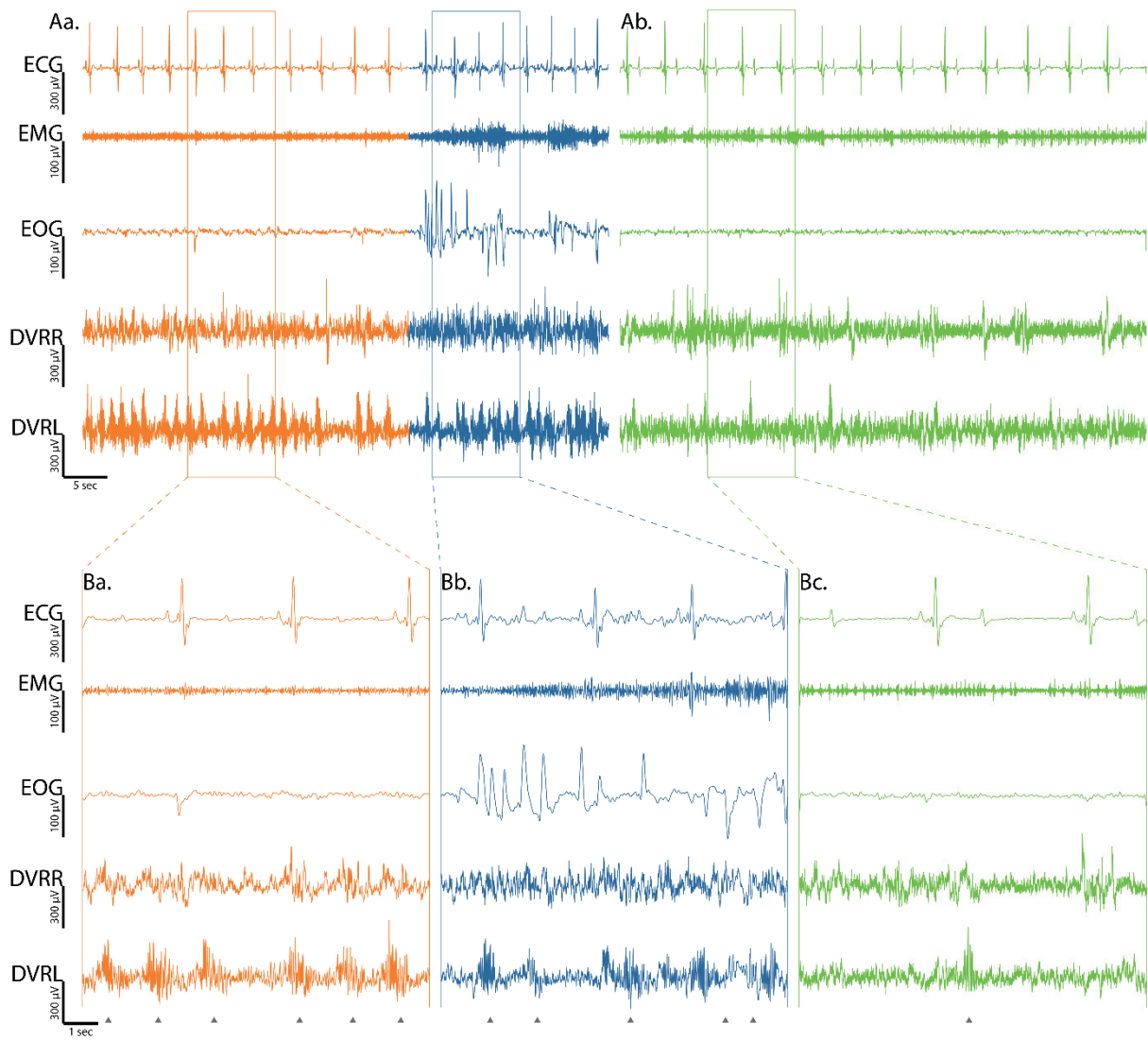


Figure 23 – Raw signal of the recorded vigilant states in the *Broadleysaurus major*. A. One minute recordings of the ECG, EMG, EOG, Right DVR LFP (DVRR) & Left DVR LFP (DVRL) during (Aa) wakefulness with QW in orange and AW in blue and (Ab) sleep (green). B. 10 second zoom of the same parameters as A during (Ba) QW, (Bb) AW, & (Bc) sleep. The grey arrowhead at the bottom show the 20Hz oscillations associated with the respiration.

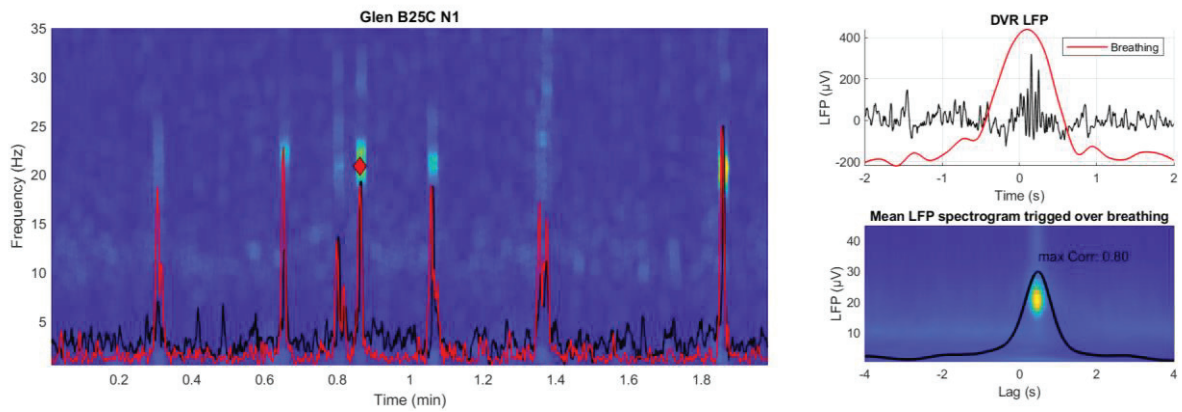


Figure 24 – LFP and Respiration correlation. The black line is the graphical representation of all the respiration detected using video analysis. The colour map is the mean power of the LFP 10 seconds before and after those events with a visible power increase (yellow spot) between 15 and 20Hz.

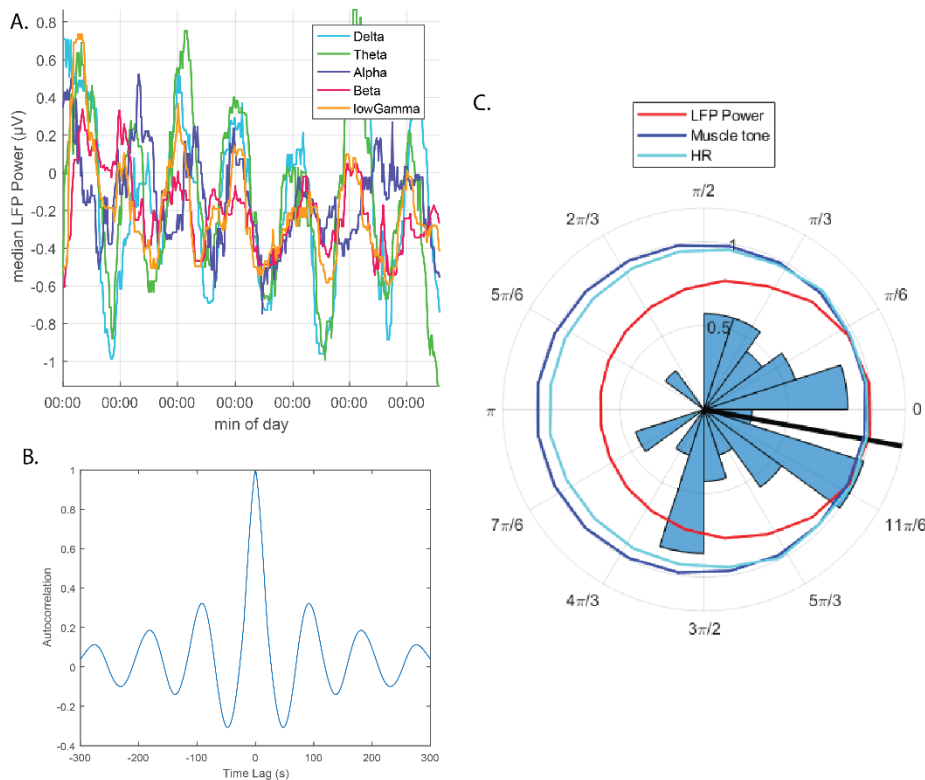


Figure 25 – A. 10min representation of the filtered data off all frequency bands during the sleeping period of one animal. B. Autocorrelation of the DVR LFP over the 12h hours of night of one animal C. Polar plot of the distribution of the eye movements within each  $\beta$  cycle; the mean eye movement preference is represented with a black line. The red line is the mean  $\beta$  power across the  $\beta$  cycle. The dark blue line is the mean muscle tone across the  $\beta$  cycle. The light blue line is the mean instantaneous Heart Rate across the  $\beta$  cycle.

## Discussion

### Agamid sleep

#### *Sleep states in Pogona vitticeps and their comparison to SWS and REMS:*

The sleep of the bearded dragon has been divided into two distinct substates with specific brain patterns (Libourel et al., 2018; Shein-Idelson et al., 2016). The first cerebral sleep state is associated with a very low amplitude of the brain activity overprinted with high voltage waves. The second state is characterised by a broader, more desynchronised brain activity similar to what could be recorded during the waking period. In this study, we demonstrated that during behavioural sleep of the bearded dragons, all physiological parameters tend to diminish compared to the waking states. Muscle tone is lower, there is a notable decrease in eye movements and their amplitude, and the heart rate decreases. When looking at the sleep state physiology, no visible differences could be established either in the evolution of the muscle tone, the heart rate, or the brain temperature. Only the eye movements, even if rare, were confirmed to be more numerous and more frequent in S2 compared to S1.

When comparing to land mammals and birds, S1 and S2 are thought to be the *Pogona* equivalent of the mammalian and avian SWS and REMS (respectively). To some extent, this holds up. S1 is characterised by slow isolated HvW that could resemble the slow wave of the SWS or the HSpW ripples complex. The physiological parameters are also significantly lower in S1 compared to wakefulness like they are in NREMS. However, as discussed earlier, an increasing amount of evidence suggest that the correspondences stop there. Even if they are less numerous, there are still eye movements occurring during S1 which very seldomly occurs in mammalian and avian SWS. The HvW that were thought to be a phenomenon restricted to S1 were identified in S2 but also during wakefulness contradicting the idea that they could be homologous to the SWP of the SWS or the HSpW ripples complex. Besides, the typical electrophysiological pattern of S1 tends to occur instantly when the animal closes the eye even when the animals display a waking posture. Moreover, our data shows that small movements of the limbs, head and tail that could be associated with twitches, which are happening in REMS in mammals and birds, also appeared during S1. For S2, the desynchronised brain activity and the higher density of eye movement suggest that it could be homologous to REMS. However, the physiology of REMS does not exist in the *Pogona vitticeps*. No muscle atonia is visible, the heart rate, and brain temperature stay unchanged, and the twitches are quite rare in this state.

#### *Stellagama stellio* sleep

Based on our results in the bearded dragon, sleep behaviour has been easily identified in a closely related species, the starred agama. The sleeping behaviour of this species is very similar to the one of the bearded dragons with the head laid down, their eyes closed, and the limbs relaxed. Their sleep period is reinforced and unique.

When looking at the brain activity, we showed that sleep in this species can be divided into two distinct states. Those sleep states are surprisingly similar to what has been recorded for

the bearded dragon with the first one marked by low brain activity except for HvW and the second one similar to the brain activity recorded in the wake period. While those results reinforce the idea that two cerebral sleep states could exist in squamates, the similarities with REMS and SWS are becoming less obvious. Like in the bearded dragons, even if the S2 does match some defining points of REMS (i.e., desynchronised brain activity, eye movements), this state also contradicts others. For example, muscle tone in this species is not significantly diminished when switching from S1 to S2. Likewise, no clear evidence of twitching has been identified in this species either. Rare limb, tail or head movements have been recorded and those few movements were not associated with one specific sleep state.

The alternance between two brain patterns found in the DVR of two different species of *Agamidae* reveal that sleep is not homogenous in squamates. The HvW particularly visible during S1, the predominance of eye movements during S2 and the similarity with the wake brain activity during this state could make us think that these states could be SWS and REMS, respectively. However, the muscle tone of the squamates during their sleep might be a key factor in our understanding of sleep states. Indeed, the muscle atonia is a defining point of REMS in mammals. By disabling any possibility of movements (Valencia Garcia, Luppi, et al., 2018), it is thought to preserve this state from the particularly vivid re-enactments of motor automatisms and save energy by contributing to reduce the energy spent at this level (Schmidt, 2014). In squamates however, atonia does not seem to exist or, at the very least, no tonus differences were recorded between S1 or S2. If we assume that S1 and S2 are respectively equivalents of SWS and REMS, why do squamates not express those motor automatisms like mammals with impaired atonia? We saw that, in mammals, this suppression led to the expression of complex behaviours mimicking ones expressed during wakefulness (Arnulf, 2012). However, almost no movements were recorded during the sleeping period of our animals, and they preferentially appeared during S1 not S2. Multiple explanations could explain this lack of movement in regard to the absence of atonia. Either the function of REMS associated with twitches does not exist in reptiles relieving the need for muscle atonia; or non-avian reptiles found other ways to prevent movements during their sleep. The first possibility could mean that muscle atonia, and its functions, and dream-like behaviour in mammals, and in parts in birds, arose later in the evolution of REMS and is potentially linked to homeothermia as poikilotherms do not express it. The second possibility suggests that squamates developed different networks to suppress movements during their sleep. Yet, as discussed in the introduction, muscle atonia networks are mainly composed of nuclei found in the pons and the medulla and those nuclei are highly conserved in the amniotes likely serving the same function. Even if it seems unlikely that squamates developed another way of blocking movements, recording those nuclei in squamates could help understand their role in sleep. Lastly, one possibility could be that squamates are atonic by default during their sleep. Muscle architecture is quite different between squamates and mammals. While the latter have muscles made of a mix of red and white fibre allowing for more enduring muscles, muscles in squamates are mostly composed of white fibres (Bonine et al., 2005). Even though those fibres are efficient for rapid anaerobic contractions, they take a lot more time to recover from an effort. In a context of predation, movement is key for survival and tired muscles are

not efficient in fight-or-flight response. Therefore, we could imagine that squamates show a “default atonia” for all their sleeping period and not just one state as it would represent a significant energy saving. One way to test this hypothesis would be to look for atonia in squamates that have a more diverse set of fibres like what has been reported in the savannah monitor (*Varanus exanthematicus*) (Moritz & Schilling, 2013). This could account for the absence of muscle tone difference between S1 and S2 but also the notable decrease between QW and the sleep states. This could also explain why so little movements were measured during sleep in our animals.

Regarding those movements, little is known about muscle twitches in squamates as they have only been reported *in ovo* so far (Corner, 1977). Our recordings showed the existence of rare movements of the limbs, tails, and head in both *Agamidae* species. Are those events twitches like those of mammals and birds? Those occurrences in adults homeotherms almost exclusively happen during REMS. They usually are jerky automatisms of the extremities and face and, according to the developmental theory, are the substrate of the sensory-motor connection refinements. If squamates night movements are slow equivalents of homeotherm twitches, they do not always occur at the right time in the alternance of the sleep states and are a lot less numerous. They appear during both states but in less than 3% of them for the starred agama and less than 17% of them for the bearded dragons. The twitches occurring during S2 could be equivalent of the mammalian ones but what about the ones occurring during S1? Are they even really sleep movements? Taken together with the occurrence of HvW as soon as the animals close their eyes, these might suggest that, while S1 meet the definition of sleep, it might also be the substrate of small awakenings. To test this hypothesis, further investigation should be conducted on arousal threshold estimation during the different sleep states and see if small movements are correlated with lower arousal thresholds. Moreover, if twitches are present *in ovo* in squamates (Corner, 1977), but quite rare once the animals are mature, this reinforces the hypothesis of the developmental theory. Their absence in adults stage could be explained by the relative maturity of the individuals when hatching, the need to refine sensori-motor connections is not as strong as in mammals and twitch number might diminish quickly over time to be almost absent in adults.

We wanted to explore whether, like mammals, significant temperature changes occur during the sleep period of our species depending on the state they are in. This study allowed us to show that in birds, as in mammals, a significant increase of the brain temperature occurred when switching from SWS to REMS (Ungurean et al., 2020). For our squamates, however, the results showed no measurable changes occurring when switching from S1 to S2. If S2 is to be considered a REMS-like state, these results go against the brain warming theory postulating that one of REMS purpose is to counteract the temperature loss during SWS to optimize awakening. This could either mean that this physiological change and its related function appeared independently in both homeotherm groups or that it is but a mere substrate of another physiological phenomenon. Recent studies using fast-ultrasound imaging (FUS) measured the brain blood flow during sleep and showed that a significant increase of blood flow occurred during REMS in mammals (Bergel et al., 2018). This phenomenon could be



responsible for the temperature increase we observe in mammals and birds by providing more heat from the animal core. It will be interesting to know if a similar phenomenon happens in squamates by using the same technique. It is commonly accepted that an increase of blood flow in a brain area equates to an increase of metabolic activity in said area. Thus, an increase of blood flow during one of the sleep states in our squamates could add some valuable information on the homology between those states and those in homeotherm.

One other point of interest of the starred agama sleep are the HvW. HvW have been described in multiple species of squamates and even some chelonians. They are thought to be the marker of sleep in those species and have been associated as an equivalent of SWP or the HSpW in *Pogona vitticeps*. However, we showed that it was not actually the case in the bearded dragon, as HvW were recorded in every vigilant state as they have been in the *Stellagama stellio*. In the latter, they varied in density, being more numerous during the waking period and the S2 compared to the S1 but were present throughout the day even in awake animals. The mechanisms behind this phenomenon are unclear and only morphological comparisons are available to us to assess it. As HvW exists outside S1 in *Pogona vitticeps* as well as in the *Stellagama stellio* and are not a marker of S1 in squamates, this raises the question of what S1 electrophysiological signal looks like without them. When looking at the overall activity in all the bands of the power spectrum, S1 in the bearded dragon and the starred agama is very quiet with nearly flat LFP detected in the DVR. Thus, even if we do not exclude that HvW are related to sleep, from our point of view, the DVR signal could be interpreted as an alternance between a signal rich in frequency content (S2 & Wake) and a nearly flat signal (S1) with HvW superimposed in all the states. In infant mammals (humans and rodents), sleep behaviour is characterised by an alternance of long neuronal silences with bursts of activity (Dereymaeker et al., 2017; Seelke & Blumberg, 2008). If squamates S1 and S2 are equivalent to mammals and birds SWS and REMS, their phenotype is closer to the one of infants than mature mammals. As discussed earlier, this could be partially explained by the neuroanatomy of squamates. Indeed, these species do not possess the same cortical architecture as mammals with only 3 layers in their very thin cortex and this might explain why their quiet sleep periods resemble the one of mammals with immature cortical structures. The recent findings about the reptilian claustrum and its role in the generation of the HvW in *Pogona vitticeps* showed that non-avian reptiles seem to possess the underlying machinery to generate oscillations that was proposed to be similar to mammalian hippocampal sharp waves ripples complex. However, the homology with mammalian delta waves or hippocampal sharp waves ripples complex is still debatable. Indeed, even if the *Pogona vitticeps* HvW are able to propagate to close pallial structures (i.e., the DVR; (Norimoto et al., 2020)), they seem unable to propagate properly to the rest of the cortex as it does in mammals (Luppi & Fort, 2019). This goes along the idea that the reptilian brain lacks the complex pallio-pallial connectivity found in birds and mammals that sustains SWP (Rattenborg, 2006) and it would explain why it may not be expressed in lizards.

Finally, one other major point of this study was the report of the same rhythmicity found in both the starred agama and the bearded dragon. Our results showed that the starred agama

(at 25°C) displayed a rhythmicity of 113 seconds during the night compared to the 122 seconds rhythmicity of the bearded dragon. Sleep ratio in this species is not as high as the ratio of the *Pogona vitticeps* with S2 representing almost 37% of their 12h sleep period but is still higher than most recorded mammals and remarkably higher compared to every bird recorded so far. Thus, this regularity is not specific only to the bearded dragon but seems to be specific of at least the *Agamidae*. No clear theory of why those species presents this type of alternance and this rhythmicity during their sleep behaviour exists. This phenomenon being totally absent in the Argentine tegu, the other lizard species with 2 recorded sleep activities, the only report of a similar behaviour was made in 1972 and 1975 in the graduate work of S. Stropes in *Uma notata* and *Sauromalus obesus* (Stropes, 1975; Stropes et al., 1972). These two species are part of the *Iguania* a sister group of the *Agamidae* and they appear to display period of broad band activity alternating with overall neuronal silencing. Sadly, those data are lacking some key information on the precise region recorded and the periodicity and evolution of this phenomenon over longer period of time. Moreover, these species belong to the *Iguania* family which was the most studied group of lizards until the recent reports and none of those studies reported any type of rhythmicity during sleep (Libourel & Herrel, 2016). There is still a lot of work to be done to properly understand why and how this unique phenomenon exists, but one hypothesis could lie in the environment of the studied species. *Pogona vitticeps* and *Stellagama stellio*, as well as *Sauromalus obesus* and *Uma notata* studied by Stropes, are all desertic species (Stropes, 1975; Stropes et al., 1972). Deserts are a harsh environment that led to a lot of unusual phenotypes and one can only assume its unique impact on sleep phenotype. There is still a lot to be learned on the *Agamidae* sleep, especially regarding the generation of the HvW and the rhythmicity of their nights. But, to close the gap between the *Iguania* and the *Agamidae* in an attempt to understand their sleep phenotype it would thus be of interest to study their closest relatives, the *Chameleoniae*. More studies should also be conducted on the impact of the environment on sleep phenotypes. Do other desert species could show the same rhythmicity as the *Agamidae*? Does it exist outside of the *Iguanidae*? Or is it a unique phenomenon reserved to this group?

*Pogona vitticeps* and unilateral brain activity: In the second part of the first study, we showed that *Pogona vitticeps* expressed sustained unilateral eye closure during the day. This phenomenon had already been reported in other species of non-avian reptiles (Kelly et al., 2015; Mathews et al., 2006) and is well characterised in birds and mammals where it is a marker of USWS. Here, we showed for the first time that this unique behaviour happened simultaneously with an asynchrony of the brain hemispheres. When the bearded dragon closes one eye, the contro-lateral hemisphere transitions from the broad awake brain activity to an electrophysiological signature similar to what we reported as S1. In aquatic mammals and birds, USWS has been associated with high environmental pressure on the individual to keep at least a partial awareness of what is going on in their environment while resting (Lyamin et al., 2008, 2016). In lizards, unilateral eye closure (UEC) has been associated with predatory pressure with animals privileging one UEC to the other in regard to their predator's position (Kelly et al., 2015; Mathews et al., 2006). Could this be the case here? Does this mean that *Pogona vitticeps* are sleeping with one eye open during the day to stay aware of potential

threats? While our results are still limited due to our recordings being for only one animal, one way to test this hypothesis further would be to expose the bearded dragon to predatory pressure and see if and how it impacts this unilateral behaviour. One other point that can be raised on that behaviour is, is it really sleep? We showed that HvW might not be a defining sign of sleep as we identified them in every vigilant state. We also discuss the possibility of small awakenings during S1 visible in small adjustment movements without modification of the brain electrophysiology. Moreover, the switch from wakefulness to unilateral S1 pattern during UEC seems to be almost instantaneous in our recordings. Is the animal really falling asleep this fast? Or is this brain activity only a substrate of eye closure as alpha is in humans? Is it possible that the bearded dragon closes its eye simultaneously with it falling asleep? In birds, the appearance of SWS is almost instantaneous when the animal closes its eyes, and it was shown that during each sleep state the bearded dragon retains its sleep behavioural phenotype even upon otherwise arousing stimulation. To answer those questions, fine characterisation of arousal threshold during all the vigilant states of *Pogona vitticeps* need to be conducted. If arousal threshold is higher in S1 compared to wakefulness regardless of the temporality of the stimulus within the S1 episodes, then S1 could be considered a sleep behaviour in the bearded dragon even during UEC. If it is not, future investigations could take an interest in determining a substrate of S1 “depth” that discriminate potential eyes closed QW to a sleeping state and thus explore if UEC is a sleeping state or not.

### ***Broadleysaurus major* : The pebble?**

*The apparent absence of biphasic sleep in Broadleysaurus major:* Our second study about the Sudan plated lizard, *Broadleysaurus major*, raised new questions about the squamate sleep. The behavioural and physiological recordings of this species showed that, like the rest of the recorded squamates, this species displayed what we consider a sleeping behaviour. The animal assumed a resting position and physiological parameters decreased (EOG & ECG). However, the muscle tone showed no real decrease when transitioning from wakefulness to sleep. This might be due to the particularly inactive phenotype of this species. During the day, we recorded very little movements usually to eat or drink. Exploratory episodes were minimal, and the resting position was usually similar to the sleeping behaviour except for the opened eyes and the position of the limbs. There is no clear hypothesis on why we did not manage to measure a noticeable difference between QW and sleep in muscle tone. This species seems to be the embodiment of the “sit & wait” strategy and the QW, muscle wise, could be marked by an extension of the “default atonia” we hypothesised earlier.

Regarding the brain activity, the LFP linked to the sleep state showed a decrease in all band frequencies when switching from wakefulness to sleep. The awake desynchronised brain activity decreased in power. However, we did not record any signs of HvW activity in this species. The only noticeable phenomenon occurring during the sleeping period of the animals were rhythmic 20 Hz oscillations appearing throughout the night. Those burst of activity revealed themselves to be linked to the breathing activity of the animal and could be found during wakefulness as well as the sleeping period. Once the respiration subtracted from the LFP signal, the clustering analysis as well as the study of the power spectrum did not reveal

any type of second electrophysiological activity during the night suggesting that no electrophysiological differences occurred during the sleep of this peculiar species. However, while no apparent dichotomy was visible like in the *Agamidae*, the autocorrelation of the signal showed a rhythmicity in the whole LFP spectrum.

*Infraslow oscillations and sleep dichotomy?* Upon closer inspection, this rhythmicity revealed itself to be a 0.01 Hz oscillation (with a 90s period) of all the band frequencies during the night. This rhythmicity is not without recalling the rhythmicity of the *Agamidae* with their oscillations between a “frequency rich” sleep state and a quieter one only marked by HvW. Could this oscillation be the equivalent of the two sleep states in the Sudan plated lizard? In mammals, a faster infraslow oscillations exists at 0.02Hz. It has notably been associated with SWS depth and memory processing (Lecci et al., 2017). This infraslow rhythm is, however, independent from the SWS and REMS alternance. Is this the case in our species? could this oscillation be a sort of vestigial form of the two sleep states in the *Broadleysaurus major*? We discussed earlier that the clocklike alternance between the two sleep states in the *Pogona vitticeps* was still a mystery as to why and how it happens. Thought to be an exception, we showed in this work that it exists outside one species and could be a trait shared by all *Agamidae*. Moreover, the preliminary results on this infraslow oscillation (ISO) of the Sudan plated lizard showed that, while the rest of the physiology seems to be unchanged by it, eye movements tend to appear preferentially during the upstate of the ISO. This physiological signature is similar to the one found in *Agamidae* S1 and S2. However, no signs of HvW were recorded in the Sudan plated lizard contrary to the *Agamidae* where those patterns were visibly influenced by their own ISO. To try and uncover the origin and role of this ISO in *Broadleysaurus major*, one lead would be to keep pushing the phylogenetic approach. The separation between the *Scincimorpha* and the rest of the studied squamates (i.e., the *Episquamata*) happened approximatively 190 MYA during the lower Jurassic (Zheng & Wiens, 2016). To try and better understand if the duality of sleep is a common ancestral feature of the amniotes or if it appeared independently in mammals and birds and in some parts of the squamates and what part the ISO play in all that, one could take an interest in looking in the group of species who arose almost 200 MYA, the *Gekkota*. This peculiar group of lizards have drawn a lot of interest due to some of its species being able to climb on “unclimbable” surfaces using weak atomic bonds (Autumn et al., 2002) but no one took interest in their sleeping behaviour.

Another approach would be a more mechanistic one, by trying to unveil the neuronal networks implicated in lizards sleep and see how those networks activity evolve throughout the night. The description of the nuclei implicated in the generation of the two sleep states in the *Agamidae* and how it relates to the ISO of the Sudan plated lizard could help us understand how and why this rhythm exists.

Finally, another question is how it relates to the mammalian ISO and to the rest of the animal kingdom. As more and more reports try to push even further down the evolutionary tree the origin of the two sleep states (Iglesias et al., 2019; Leung et al., 2019; Medeiros et al., 2021; Tainton-Heap et al., 2020), the question of the homologies between those species and their states becomes more present and the ISO could be an investigation starting point. Could this

oscillation be the substrate of the two sleep states? How does it relate to the rest of the squamates? Does it exist in those species displaying a “paradoxical kind of sleep”? Those questions could be answered by continuing the effort in recording sleep in an even greater number of species of squamates but also outside of the amniotes group, like amphibians, that could give us critical information on the evolution of sleep architecture in the rest of the animal kingdom.

### **Study impact and limitations**

On a final note, this work has some limitation that need to be addressed. The number of individuals is limited notably on the part on the unilateral brain asymmetry of the bearded dragon. Work is to be done to increase the number of individuals recorded and confirm some of the results presented here. Moreover, to continue to better understand the evolution of sleep in squamates, new species of lizards and snakes should be studied. Our present results showed that even if sleep seems quite homogenous in one group, it might drastically differ in another (Table 1). One beginning approach would be to characterise at least one species of each group of the *Squamata* order. Regarding the results of the second study, proving that a phenomenon does not exist is a lot harder than providing it does. Therefore, to confirm our results on the Sudan plated lizard, future investigations should take particular interest on the remaining parameters left out of this work like properly evaluating the arousal threshold during the different vigilant states of the animals or measure the evolution of blood flow during their sleeping behaviour. Those parameters could help confirm the results exposed here. Finally, this work has been possible thanks to the miniaturisation and portability of equipment once limited to highly controlled environments. This allowed us to record our animals in freely moving conditions and adapt their environment to their need. However, we talked earlier on how little the Sudan plated lizard moved in our experimental setup. This is not the case in the wild. This species is quite active when meet outside of the laboratory conditions favouring the foraging approach to the sit & wait strategy. This notable difference might be due to the fact that a majority of reptiles available in pet shops are wild caught. Studies have shown a significant difference in the expression of sleep and sleep quantities when comparing species in wild versus in controlled environment. This seems to be the case here with our lizards. If we want to get the full picture on reptiles sleep and its physiological manifestation, we have to continue the effort to take the science outside the lab. The growing interest in more ecological set ups is set to help understand a phenomenon as complex as sleep. Without understanding how sleep works and evolved in response to environment constraints a lot of capital information are going to be left out. We need to keep pushing for the development of technologies that help record the animals in their environment with minimal interference in their life.

### **Conclusion**

In this work, we documented that sleep in squamates is not a simple phenomenon. The parallel between mammalian and avian sleep and sleep in squamates seems to be less and less obvious. In the first chapter of my thesis, we detailed the arguments that lead to us to

believe that, while there is a high chance that two sleep states do exist in squamates, the search for a perfect match with the mammalian definition could delay or impair our understanding of squamate sleep and thus sleep in general. The origin of the sleep dichotomy seems to be at least as old as the squamates either as two discrete states or on the ISO continuum, but the definition of their sleep should consider that they diverge millions of years ago from the species that first defined REMS and SWS, mammals and birds. In the second part of my work, we explore how the two sleep states expressed themselves in different species of squamates. In the *Agamidae*, it has been shown that the *Pogona vitticeps* display an unusual phenotype of sleep with highly rhythmic brain pattern repeating throughout the night. Here, we showed that this phenomenon is not as unique as what was thought as it was also identified in a closely related of the bearded dragon, the starred agama. While it confirmed that two sleep states are present in some squamates, it asks the question of how far the *Agamidae* rhythmicity goes. The Sudan plated lizard was the pebble in our story's shoe or so we thought. As no evident biphasic sleep was found in this species, it reopened the question of its origin in the rest of the studied squamates. But the discovery of its infraslow rhythm suggested that the answer might be even more complex. The squamates are at a key evolutionary point when it comes to sleep, and this unique group of species could hold answers on our sleep and its functions. But millions of years of evolutions have buried and fragmented those answers. It is now up to us to continue our effort in putting the pieces together to understand squamate sleep architecture and phenotypes and get a glimpse of what sleep could be. By continuing to search for a less Manichean approach of sleep, I do believe that it would help understanding our own sleep dichotomy and that understanding how and when all those patterns coevolved would bring us more than searching for the perfect sleep that meets all components of the mammalian definition of it.

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	Mammals°	<i>Pogona vitticeps</i>	<i>Stellagama stellio</i>	<i>Broadleysaurus major</i>	<i>Salvator merianae*</i>	
2 clusters during sleep behaviour	Yes	Yes	Yes	No	Yes	
Rhythmicity/Infraslow oscillation	Only during SWS	Yes	Yes	Yes	Not found	
Mammalian SWS vs Squamates Low Beta (compared to wake)						
	LFP	Slow Wave	HvW	HvW	Reduced amplitude	HvW
	Eye Mvt	No	Rare isolated EM	Rare isolated EM	Rare isolated EM	Rare isolated EM
	Muscle tone	Diminished	Diminished	Diminished	Diminished	Diminished
	Heart Rate	Diminished	Diminished	?	Diminished	Diminished
	Brain T°C	Unchanged	Unchanged	?	?	?
	Twitches	No	Yes	Yes	?	Yes
Mammalian REMS vs Squamates High Beta (compared to the other sleep state)						
	LFP	Wake-like, Theta (rodents)	Wake-like + HvW	Wake-like + HvW	Increased amplitude	15 Hz oscillation at 30°C
	EOG	REM	Some Isolated EM	Some Isolated EM	Some Isolated EM	Some Isolated EM
	EMG	Atonia	Unchanged	Unchanged	Unchanged	Reduced
	ECG	Deregulated	Unchanged	X	Unchanged	Unchanged
	Brain T°C	Increase	Unchanged	X	?	?
	Twitches	Yes	Yes	Yes	?	Yes

Table 1 –Comparative table of the states of different squamates species and the mammalian SWS and REMS if we consider the squamates LFP sleep variations to be equivalent to the mammalian sleep states. The sleep states of the squamates can be divided as low (S1) and high (S2) beta (10-30Hz) power. ° see Introduction, \* Libourel et al., 2018.

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Supplementary I – Comparative Perspectives that Challenge Brain Warming as  
the Primary Function of REM Sleep

Gianina Ungurean\*, Baptiste Barrillot\*, Dolores Martinez-Gonzalez, Paul-Antoine Libourel,  
Niels Rattenborg

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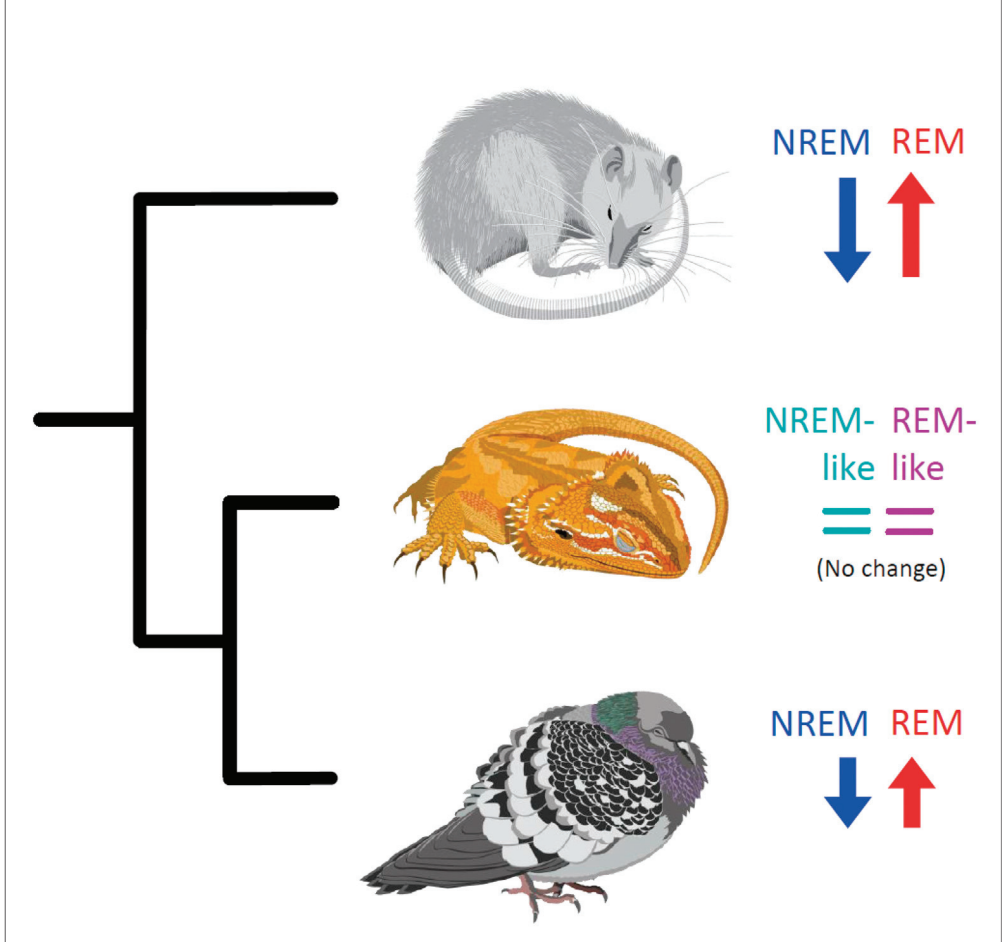
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\* These authors contributed equally

Article

# Comparative Perspectives that Challenge Brain Warming as the Primary Function of REM Sleep

## Sleep State-related Changes in Brain Temperature



Gianina Ungurean,  
Baptiste Barrillot,  
Dolores Martinez-  
Gonzalez, Paul-  
Antoine Libourel,  
Niels C.  
Rattenborg

pa.libourel@cns.fr (P.-A.L.)  
rattenborg@orn.mpg.de  
(N.C.R.)

**HIGHLIGHTS**

In many mammals, the brain cools during non-REM sleep and warms during REM sleep

Pigeons exhibit similar changes in cortical temperature during non-REM and REM sleep

Brain temperature does not increase during REM-like sleep in bearded dragon lizards

Brain warming is not a universal outcome of sleep states with wake-like brain activity

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## Article

## Comparative Perspectives that Challenge Brain Warming as the Primary Function of REM Sleep

Gianina Ungurean,<sup>1,2,3</sup> Baptiste Barrillot,<sup>2,3</sup> Dolores Martinez-Gonzalez,<sup>1</sup> Paul-Antoine Libourel,<sup>2,\*</sup> and Niels C. Rattenborg<sup>1,4,\*</sup>

## SUMMARY

**Rapid eye movement (REM) sleep is a paradoxical state of wake-like brain activity occurring after non-REM (NREM) sleep in mammals and birds. In mammals, brain cooling during NREM sleep is followed by warming during REM sleep, potentially preparing the brain to perform adaptively upon awakening. If brain warming is the primary function of REM sleep, then it should occur in other animals with similar states. We measured cortical temperature in pigeons and bearded dragons, lizards that exhibit NREM-like sleep and REM-like sleep with brain activity resembling wakefulness. In pigeons, cortical temperature decreased during NREM sleep and increased during REM sleep. However, brain temperature did not increase when dragons switched from NREM-like to REM-like sleep. Our findings indicate that brain warming is not a universal outcome of sleep states characterized by wake-like activity, challenging the hypothesis that their primary function is to warm the brain in preparation for wakefulness.**

## INTRODUCTION

Sleep is a mysterious state of reduced environmental awareness found in animals ranging from jellyfish to humans (Joiner, 2016; Libourel and Herrel, 2016; Blumberg and Rattenborg, 2017; Nath et al., 2017; Anafi et al., 2019; Iglesias et al., 2019; Kelly et al., 2019). Sleep in mammals is composed of two states, non-rapid eye movement (NREM) sleep and REM sleep, typically distinguished from one another and wakefulness by changes in brain and muscle activity. NREM sleep is characterized by the slow alternation between periods of cortical neuronal silence (down-states) and periods with high firing rates (up-states) that collectively give rise to high-voltage slow waves (0.5–4 Hz) in the electroencephalogram (EEG) (Timofeev et al., 2001; Vyazovskiy et al., 2009). REM sleep is typically characterized by continuous high firing rates comparable to wakefulness (Evarts, 1962; Mukhametov and Stokova, 1977; Timofeev et al., 2001; Vyazovskiy et al., 2009; reviewed in Jones, 2016), resulting in low-voltage, high-frequency EEG activity. In addition, muscle tone is reduced during NREM sleep, when compared with wakefulness, and absent during REM sleep (Peever and Fuller, 2017). Nonetheless, brief muscle twitches occur during REM sleep resulting in movements, such as rapid eye movements. The patterns of brain activity that characterize NREM and REM sleep have been implicated in various forms of synaptic plasticity (Chauvette et al., 2012; Tononi and Cirelli, 2014; Boyce et al., 2016; Li et al., 2017; Klinzing et al., 2019), and twitching during REM sleep is thought to play a role in mapping the sensorimotor cortex during development and possibly adulthood (Dooley et al., 2020; Blumberg et al., 2020).

In addition to brain and muscle activities, other physiological processes also differ between NREM and REM sleep in mammals. Notably, in most mammals examined, cortical and sub-cortical brain temperature ( $T_{br}$ ) decreases during NREM sleep and increases during REM sleep (Kawamura and Sawyer, 1965; Hayward and Baker, 1969; Kovalzon, 1973; Obal et al., 1985; Wehr, 1992; Franken et al., 1992; Gao et al., 1995; Landolt et al., 1995; Csernai et al., 2019; Hoekstra et al., 2019; Komagata et al., 2019; see Hayward and Baker, 1968 and Hayward and Baker, 1969 for conflicting results in rhesus monkeys [*Macaca mulatta*]). Brain warming during REM sleep is thought to result from an increase in blood flow from the warmer body core to the brain to support this activity (Denoyer et al., 1991; Wehr, 1992; Parmeggiani, 2007; Pastukhov and Ekimova, 2012; Bergel et al., 2018). In this regard, brain warming might simply be a functionless by-product of functions that require increased neuronal activity, such as brain development and other types of synaptic plasticity. Alternatively, it has been proposed that increased neuronal activity is the mechanism the sleeping brain employs to periodically warm itself (Wehr, 1992; Lyamin et al., 2018). According to this hypothesis,

<sup>1</sup>Avian Sleep Group, Max Planck Institute for Ornithology, 82319 Seewiesen, Germany

<sup>2</sup>CRNL, SLEEP Team, UMR 5292 CNRS/U1028 INSERM, Université Claude Bernard Lyon 1, Lyon, 69675 Bron, France

<sup>3</sup>These authors contributed equally

<sup>4</sup>Lead Contact

\*Correspondence: pa.libourel@cns.fr (P.-A.L.), rattenborg@orn.mpg.de (N.C.R.)

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brain warming is beneficial because it counteracts cooling occurring during preceding NREM sleep and thereby prepares the animal to awaken and rapidly interact adaptively with the environment (Wehr, 1992; Lyamin et al., 2018). The brain warming hypothesis remains largely untested, and it is unclear whether the brain warms during similar sleep states in other taxonomic groups.

Birds exhibit NREM and REM sleep characterized by changes in EEG activity similar to those observed in the mammalian cortex, despite differences in the organization of homologous neurons. In contrast to the laminar organization of neurons, with apical dendrites spanning the layers of the mammalian cortex, developmentally and functionally homologous (“cortical”) regions of the avian brain, such as the visual hyperpallium (homolog of the mammalian primary visual cortex) (Medina and Reiner, 2000; Güntürkün et al., 2017; Briscoe and Ragsdale, 2018; Stacho et al., 2020), are composed of nuclear structures consisting of densely packed stellate neurons (Olkowicz et al., 2016). Similar nuclear structures comprise the dorsal ventricular ridge (DVR), a large brain region only found in sauropsids (birds and non-avian reptiles), involved in performing cortex-like functions (Güntürkün et al., 2017). Despite this fundamental difference in cytoarchitecture, as in mammals, an increase in EEG and local field potential (LFP) slow waves (approximately 1–5 Hz with a peak at 2 Hz) in the hyperpallium distinguishes NREM sleep from wakefulness and REM sleep in birds (van der Meij et al., 2019). As in mammals (Massimini et al., 2004; Huber et al., 2004), NREM slow waves travel through the hyperpallium (van der Meij et al., 2019) and are homeostatically regulated in a local use-dependent manner in pigeons (*Columba livia*) (Lesku et al., 2011b; Rattenborg et al., 2019). In addition to wake-like EEG activity, as in mammals, avian REM sleep is characterized by (at least partial) reductions in muscle tone (Dewasmes et al., 1985; Rattenborg et al., 2019); twitching, including rapid eye movements; suppressed thermoregulatory responses (Heller et al., 1983; Scriba et al., 2013); and its prevalence in young altricial birds (Scriba et al., 2013). One notable difference between birds and mammals is the short duration (typically <10 s) of bouts of REM sleep in birds (Tisdale et al., 2018b).

Interestingly, NREM-like and REM-like sleep were recently described in bearded dragon (*Pogona vitticeps*) lizards (Shein-Idelson et al., 2016; Libourel et al., 2018; Norimoto et al., 2020). NREM-like sleep was characterized by high-voltage slow LFP “sharp-waves” in the DVR, and REM-like sleep was characterized by LFP activity resembling wakefulness and eye movements occurring under closed eyelids. Although recent work has implicated the claustrum in the genesis of NREM sleep slow waves in mice and the slow “sharp waves” in bearded dragons, the claustrum appears to generate these field potentials via different mechanisms; in mice, the claustrum coordinates the synchronous inhibition of cortical neurons across several cortical areas, resulting in the down-state of slow waves (Narikiyo et al., 2020), whereas in bearded dragons, bursts of neuronal activity (giving rise to LFP sharp waves) propagate as traveling waves of excitation from the claustrum to the rest of the pallial DVR (Norimoto et al., 2020; see also Tisdale et al., 2018a). Given this apparent difference and the fact that not all of the components that typically characterize REM sleep in mammals and birds have been established in bearded dragons, we refer to these states as *NREM-like* and *REM-like* sleep (Libourel and Barrillot, 2020).

As in mammals, during REM sleep in pigeons and REM-like sleep in bearded dragons, hyperpallial and DVR neurons, respectively, fire at an elevated rate when compared with preceding sleep (Shein-Idelson et al., 2016; van der Meij et al., 2019), and therefore might cause  $T_{br}$  to increase via increased blood flow from the animal’s warmer core. However, few studies have examined sleep state-related changes in  $T_{br}$  in birds, and none have examined bearded dragons. In birds, four studies examined hypothalamic temperature and one measured temperature in the nidopallium, a structure in the DVR. Graf et al. (1987) present a 4-h plot of hypothalamic temperature occurring during wakefulness, NREM sleep, and REM sleep in pigeons recorded at 10°C ambient temperature, but hypothalamic temperature did not vary systematically with state. In rooks (*Corvus frugilegus*) recorded at 8°C and 15°C, hypothalamic temperature declined during NREM sleep, but a significant increase was not detected during REM sleep (Szymczak et al., 1989). By contrast, Pastukhov et al. (2001) reported decreases and increases in hypothalamic temperature during NREM and REM sleep, respectively, in pigeons recorded at 21°C. It is unclear whether these differences are due to ambient temperature or the temporal resolution and sensitivity of the thermistors used in the respective studies. Hypothalamic temperature was also measured in sleeping ostriches (*Struthio camelus*), but the temporal resolution (once every 2 min) of the thermistor was too low to identify sleep state-related changes in temperature (Lesku et al., 2011a). In pigeons recorded at 22°C, temperature declined during NREM sleep, but a consistent change in temperature was not detected during REM sleep in the nidopallium (Van Twyver and Allison, 1972). Given that variations in  $T_{br}$  are smaller near the surface of the brain in

chickens (*Gallus gallus domesticus*) (Aschoff et al., 1973), the thermistor may not have been sensitive enough to detect changes in temperature during REM sleep in the nidopallium, a more superficial structure than the hypothalamus. Thus, apart from one study of hypothalamic temperature in pigeons, a consistent increase in temperature has not been detected during REM sleep in the birds examined. Moreover, the study reporting an increase in hypothalamic temperature during REM sleep did not characterize how temperature changed throughout and following bouts of REM sleep of different durations. It is also unknown whether temperature increases during REM sleep in “cortical” portions of the avian brain, an apparent requisite for brain warming to have a positive impact on cognitive performance upon awakening (Wehr, 1992). Finally, it is unknown whether  $T_{br}$  varies with the type of sleep in bearded dragons. To address these questions, and thereby expand our understanding of  $T_{br}$  regulation during sleep in non-mammals, we examined sleep state-related changes in temperature in the visual hyperpallium of pigeons and DVR of bearded dragons.

## RESULTS

Across the night, pigeons experienced nearly 1,000 ( $979.6 \pm 100.4$ ) bouts of REM sleep lasting  $6 \pm 0.6$  s (range,  $1\text{--}30.7 \pm 7.1$  s,  $N = 7$ ). Figures 1A and 1B illustrate the relationship between brain state and changes in  $T_{br}$  on 90- and 10-min time scales. Decreases and increases in  $T_{br}$  were related to the occurrence of NREM and REM sleep, respectively, with the peak  $T_{br}$  associated with a bout of REM sleep occurring after the bout ended (Figure 1B). To characterize the precise time course of  $T_{br}$  change during bouts of NREM and REM sleep of varying durations (Figures 1C and 1D), we examined isolated bouts of each state. Longer bouts of NREM sleep resulted in greater decreases in  $T_{br}$  (linear mixed effect model, Table S1;  $\beta_0$  [intercept]  $\pm$  SE =  $-7.30 \times 10^{-3} \pm 8.70 \times 10^{-4}$ ,  $p = 1.56 \times 10^{-4}$ ;  $\beta$  (slope)  $\pm$  SE =  $3.11 \times 10^{-3} \pm 3.31 \times 10^{-4}$ ,  $p = 8.55 \times 10^{-5}$ ) (Figures 1C and 2A) and longer bouts of REM sleep resulted in greater increases in  $T_{br}$  ( $\beta_0 \pm$  SE =  $7.86 \times 10^{-3} \pm 5.70 \times 10^{-4}$ ,  $p = 6.48 \times 10^{-6}$ ;  $\beta \pm$  SE =  $3.37 \times 10^{-4} \pm 5.59 \times 10^{-5}$ ,  $p = 1.04 \times 10^{-3}$ ) (Figures 1D and 2B).  $T_{br}$  peaked on average 9 s after a bout of REM sleep, regardless of bout duration ( $\beta_0 \pm$  SE =  $9.22 \pm 1.93$ ,  $p = 3.05 \times 10^{-3}$ ;  $\beta \pm$  SE =  $3.37 \times 10^{-4} \pm 5.59 \times 10^{-5}$ ,  $p = 0.1$ ) (Figures 1D and 2C). This hysteresis accounts for the increase in  $T_{br}$  during short episodes of NREM sleep preceded by REM sleep (Figure 1B). Finally, we found that REM sleep was more likely to occur (density per 15 s) at lower  $T_{br}$  ( $\beta_0 \pm$  SE =  $2.18 \pm 0.18$ ,  $p = 2.21 \times 10^{-5}$ ;  $\beta \pm$  SE =  $-0.50 \pm 0.08$ ,  $p = 1.02 \times 10^{-3}$ ) (Figure 2D).

In bearded dragons, brain (DVR) activity alternated between NREM-like and REM-like sleep throughout most of the night (Figure 3A). The bearded dragons experienced  $436 \pm 133.1$  bouts of REM-like sleep lasting  $49 \pm 12.8$  s (range,  $8.8 \pm 6.3\text{--}209.2 \pm 81.9$  s,  $N = 4$ ).  $T_{br}$  either showed little change or decreased across the night (Figure S1). Despite exhibiting bouts of REM-like sleep that lasted on average 8.2 times longer than bouts of REM sleep in pigeons, average  $T_{br}$  did not increase when the bearded dragons switched from NREM-like to REM-like sleep (Figures 3B and S1).

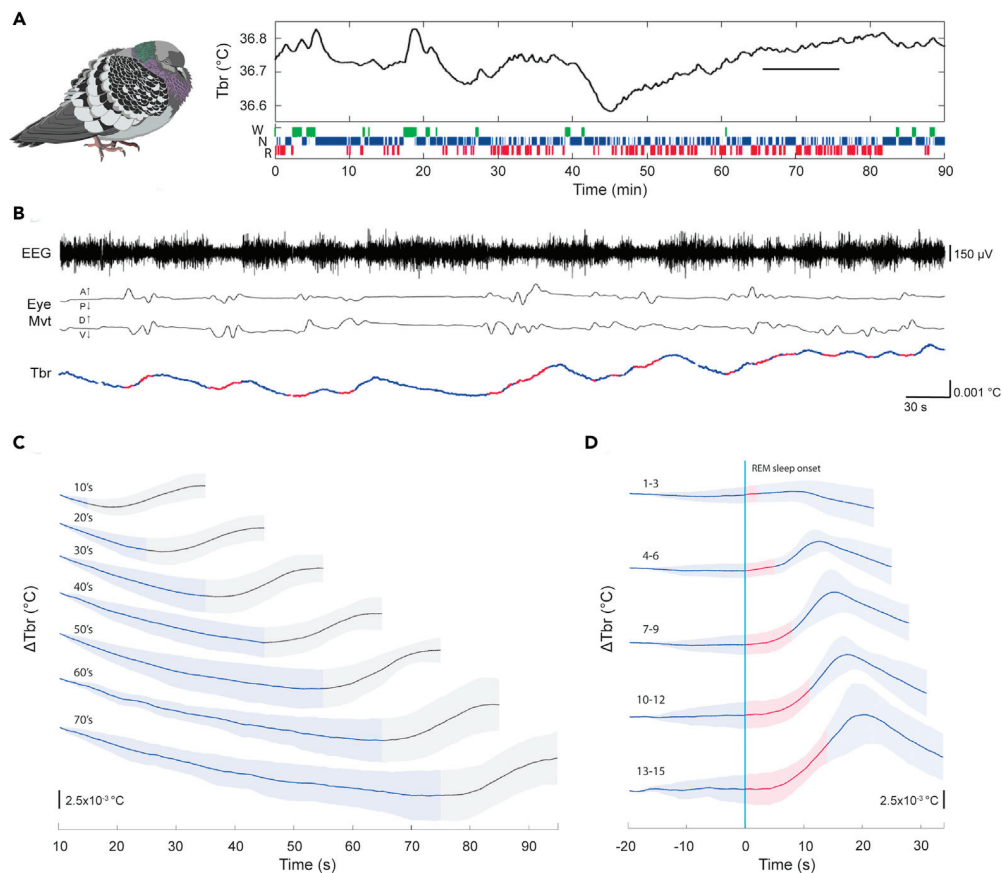
## DISCUSSION

It has been proposed that brain warming during REM sleep enhances performance upon awaking (Wehr, 1992). For this to be true, warming should occur in sub-cortical and cortical structures, as adaptive performance likely depends on the entire brain. Our results demonstrate that “cortical” warming occurs during REM sleep in pigeons, but not during a REM-like sleep in bearded dragons.

In pigeons, temperature in the visual hyperpallium decreased during NREM sleep and increased during REM sleep. Although this pattern is consistent with one study of hypothalamic temperature in pigeons, the average maximum increase was 10 times less in the hyperpallium (see Figure 1A in, Pastukhov and Eki-mova, 2012). The smaller change in temperature in the hyperpallium, the most dorsal part of the avian brain, is consistent with the observation that although temperature covaries throughout the brain in awake and sleeping chickens, it varies less near the dorsal surface (Aschoff et al., 1973). Also, the small magnitude of state-related changes in  $T_{br}$  might explain why a consistent increase in nidopallial temperature was not detected during REM sleep in the earlier study of pigeons (Van Twyver and Allison, 1972), as the average maximum increase in  $T_{br}$  ( $0.01^\circ\text{C}$ ) in the hyperpallium is equivalent to the minimum sensitivity of the thermistor used to measure temperature in the nidopallium.

Although the sleep-state-dependent changes in  $T_{br}$  in pigeons are qualitatively similar to those observed in several mammals, the magnitude is much smaller. For example, the rate of cooling during NREM sleep and the rate of warming during REM sleep are approximately 10–20 and 5–12 times slower, respectively, than in





**Figure 1. Brain Temperature ( $T_{br}$ ) during NREM and REM Sleep in Pigeons**

(A)  $T_{br}$  dynamics related to brain state across a 90-min period in a pigeon. Hypnogram (bottom plot) of brain state showing wakefulness (W, green), rapid eye movement (REM) sleep (R, red), and NREM sleep (N, blue). Note the frequent short bouts of REM sleep.

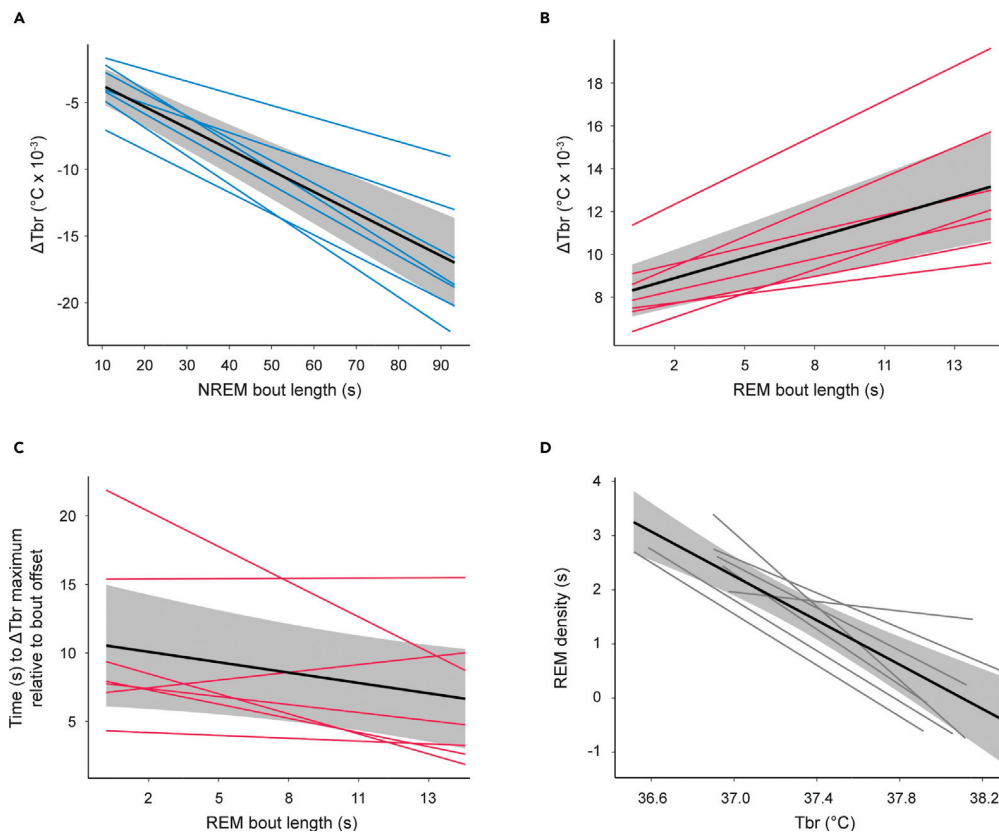
(B) Expanded 10-min period, demarcated by the bar in (A), showing electroencephalogram (EEG) activity and  $T_{br}$ , both recorded from the hyperpallium, and eye movements during NREM and REM sleep, color coded as in (A) on the bottom  $T_{br}$  recording. Lower-amplitude EEG activity and eye movements occur during REM sleep. Eye Mvt: left eye movements in the vertical (D, dorsal; V, ventral) and horizontal (A, anterior; P, posterior) planes determined from pupillometry.

(C) Longer bouts of NREM sleep are associated with greater brain cooling. Lines show the change in  $T_{br}$  (mean  $\pm 1$  standard deviation) relative to  $T_{br}$  10 s after NREM sleep onset for NREM sleep bouts in 10-s duration categories (i.e., 10's = 10–19 s; 20's = 20–29 s; etc.). The 20 s after the middle of each category is plotted in gray, as it includes a mixture of the terminating state (wakefulness or REM sleep) and any subsequent states.

(D) Longer bouts of REM sleep are associated with greater brain warming. Lines show the change in  $T_{br}$  (mean  $\pm 1$  standard deviation) during 20 s of NREM sleep (blue) before REM sleep onset (vertical blue bar), during bouts of REM sleep (red) in 3-s duration categories (i.e., 1–3 s, 4–6 s, etc.), and during 20 s of NREM sleep following REM sleep offset, defined as the middle of each duration category. For each duration category,  $T_{br}$  is plotted relative to  $T_{br}$  20 s before REM sleep onset. Pigeon illustration by Diamond Kylo.

rodents (Franken et al., 1992; Hoekstra et al., 2019). Moreover, the maximum average temperature increase associated with long (13–15 s) bouts of REM sleep, including the 9-s lag after REM sleep offset, is only 0.01°C in the hyperpallium. The reasons for this difference are unknown, but might include insulation (feathers versus hair), the level of neuronal activity and associated blood flow in the respective states, and the regulation of body temperature and the temperature of blood reaching the brain (Porter and Witmer, 2016).

In contrast to the sleep state-related changes in  $T_{br}$  in pigeons, DVR temperature did not increase when bearded dragons switched from NREM-like to REM-like sleep. Based on research in mammals, the absence

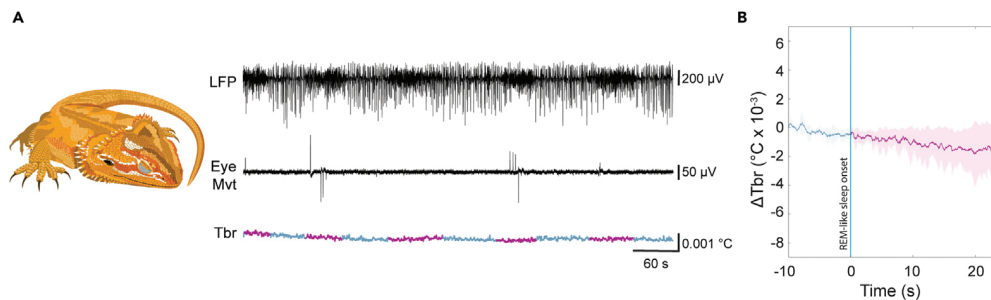


**Figure 2. Relationships between Sleep States and Brain Temperature ( $T_{br}$ )**

(A and B) Relationship between (A) NREM and (B) REM sleep bout length and change in brain temperature in pigeons. y Axis values are the peak decreases and increases in  $T_{br}$  associated with bouts of NREM and REM sleep, respectively. (C) Relationship between REM sleep bout length and the time to the peak maximum  $T_{br}$  relative to the bout offset. (D) Relationship between REM sleep density (seconds per 15 s, see [Methods](#)) and  $T_{br}$ . Each plot shows the regressions for each pigeon (in blue and red for NREM and REM sleep, respectively) and the estimates (in black) with 95% confidence intervals (gray).

of brain warming might be due to blood flow to the brain. Either the increase in neuronal firing rate during REM-like sleep does not induce changes in blood flow or blood flow does increase, but a sufficient temperature gradient does not exist between the brain and body core for this flow to cause brain warming. In this regard, it would be important to know if dragons retain more heat acquired through basking and activity in the body than the head, due to the lower surface area to volume ratio of the body. It would also be informative to determine whether blood flow increases, as shown in rats ([Bergel et al., 2018](#)), during REM-like sleep in bearded dragons.

Our findings are consistent with the notion that sleep state-related changes in temperature are a trait unique to endotherms. However, more species of birds, as well as mammals, need to be examined to determine whether this is a general pattern. In addition, more reptiles need to be examined, especially given that the changes in brain activity that define sleep states in bearded dragons have not been found in all reptiles examined ([Libourel and Herrel, 2016](#); [Libourel et al., 2018](#)). It would also be interesting to examine small and large reptiles, as  $T_{br}$  during sleep might depend on a reptile's size-dependent ability to retain core heat acquired through basking and physical activity ([Tattersall, 2016](#)). Investigating sleep and  $T_{br}$  in tegu lizards (*Salvator merianae*) that exhibit facultative endothermy during the reproductive season ([Tattersall et al., 2016](#); [Tattersall, 2016](#)) or in Galapagos marine iguanas (*Amblyrhynchus cristatus*) that undergo periods of cooling while feeding at sea ([Bartholomew, 1966](#); [Butler et al., 2002](#)), would also be informative. Finally,  $T_{br}$  should also be examined in other ectothermic animals, such as zebrafish and



**Figure 3. Brain Temperature ( $T_{br}$ ) during NREM-like and REM-like Sleep in Bearded Dragons**

(A) 10-min local field potential (LFP) recording from the dorsal ventricular ridge (DVR) of a sleeping bearded dragon showing state-related changes in brain activity. NREM-like sleep is characterized by higher LFP amplitude and  $\delta$  [0.5–4 Hz]/ $\beta$  [11–30 Hz] ratio, when compared with REM-like sleep. The brain states are color coded (NREM-like sleep, cyan; REM-like sleep, magenta) in the bottom  $T_{br}$  recording. The electrooculogram (Eye Mvt) shows the association between eye movements and brain state.

(B)  $T_{br}$  recorded from the DVR of sleeping bearded dragons 10 s before and 30 s after the transition (vertical blue bar) between NREM-like and REM-like sleep.  $T_{br}$  values (mean  $\pm$  1 standard deviation; see Figure S1 for individual data) are expressed relative to  $T_{br}$  10 s before the transition. Note the absence of an increase in  $T_{br}$  following the transition between sleep states. The slight drop in  $T_{br}$  across the two states reflects the decline in  $T_{br}$  across the night (Figure S1). Bearded dragon illustration by Damond Kyllö.

cephalopods, wherein REM-like sleep states have been described (Leung et al., 2019; Iglesias et al., 2019).

Our findings contribute to, but do not resolve, the debate over proposed homology between REM-like sleep in bearded dragons and REM sleep in mammals and birds (Libourel and Barrillot, 2020). The interpretation of our findings depends on the emphasis given to specific components of REM sleep present in mammals and birds (Blumberg et al., 2020). If an increase in  $T_{br}$  is considered an essential feature of REM sleep, then our findings suggest that REM-like sleep in bearded dragons is not homologous to REM sleep in mammals and birds, and that REM sleep evolved independently in mammals and birds, perhaps in association with the independent evolution of endothermy. Alternatively, it is possible that the states are homologous and brain warming during REM sleep secondarily emerged independently in mammals and birds. Clearly, further studies are needed to define the essential components of REM sleep and their evolution (Blumberg et al., 2020; Libourel and Barrillot, 2020).

Brain warming might be an unimportant or even costly epiphenomenon of REM sleep in endotherms or an advantageous component of this state. According to the brain warming hypothesis, warming during REM sleep counteracts cooling during prior NREM sleep, and thereby prepares the animal to rapidly interact adaptively with its surroundings upon awakening (Wehr, 1992; Lyamin et al., 2018). The brain warming hypothesis is challenged by several lines of evidence (Gao et al., 1995; Lima et al., 2005; Ungurean and Rattenborg, 2018). Notably, mammals and birds sleeping in riskier situations suppress REM sleep (Lesku et al., 2008; Gravett et al., 2017; Lyamin et al., 2018; Tisdale et al., 2018a, 2018b), which, according to the brain warming hypothesis, would render them less likely to respond adaptively to a threat upon awakening. Nonetheless, it is worth considering the implications that our findings have for this hypothesis. In pigeons, brain cooling during NREM sleep and warming (albeit small) during REM sleep, as well as the increased density of REM sleep when  $T_{br}$  is low, are consistent with the brain warming hypothesis. However, even though ecologically important behaviors, such as courtship song in male zebra finches (*Taeniopygia guttata*), can occur faster at naturally occurring higher brain temperatures (Aronov and Fee, 2012), behavioral tests are needed to determine whether the small increase in  $T_{br}$  during REM sleep in pigeons has an ecologically meaningful impact on performance upon awakening. Finally, regardless of whether REM-like sleep in bearded dragons is homologous to REM sleep in mammals and birds, the absence of brain warming suggests that the primary function (if one exists) of periods of increased neuronal activity during sleep is not to warm the brain. Other processes linked to increased neuronal activity, such as learning, memory consolidation, and

brain development, are likely candidates for a shared function of increased neuronal activity during sleep.

### Limitations of the Study

Our study has several limitations that should be taken into consideration. As we only studied pigeons and bearded dragons, more species need to be examined to determine whether our findings apply to other birds and reptiles. Additional studies are also needed to determine whether the relationship, or lack thereof, between brain state and  $T_{br}$  depends on ambient temperature and the animal's energetic status. The impact that REM sleep-related brain warming has on waking performance should also be examined in pigeons. Finally, additional research on bearded dragons is needed to establish the extent to which NREM-like and REM-like sleep are homologous to NREM and REM sleep in mammals and birds.

### Resource Availability

#### Lead Contact

Further information and requests should be directed to and will be fulfilled by the Lead Contact, Niels C. Rattenborg ([rattenborg@orn.mpg.de](mailto:rattenborg@orn.mpg.de)).

#### Materials Availability

This study did not generate new unique reagents.

#### Data and Code Availability

The brain temperature and pre-processed data used in this study are available at <https://edmond.mpg.de/imeji/collection/cWlHbLzSs1mhNKQ>.

## METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.101696>.

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## AUTHOR CONTRIBUTIONS

G.U., P.-A.L., and N.C.R. designed the pigeon experiment. G.U. and D.M.-G. performed the pigeon surgeries. In consultation with P.-A.L. and N.C.R., G.U. conducted the experiment and analyzed the pigeon data. B.B. and P.-A.L. designed the bearded dragon experiment, performed the surgeries, conducted the experiment, and analyzed the data. G.U., B.B., P.-A.L., and N.C.R. contributed to writing the manuscript, and D.M.-G. provided comments.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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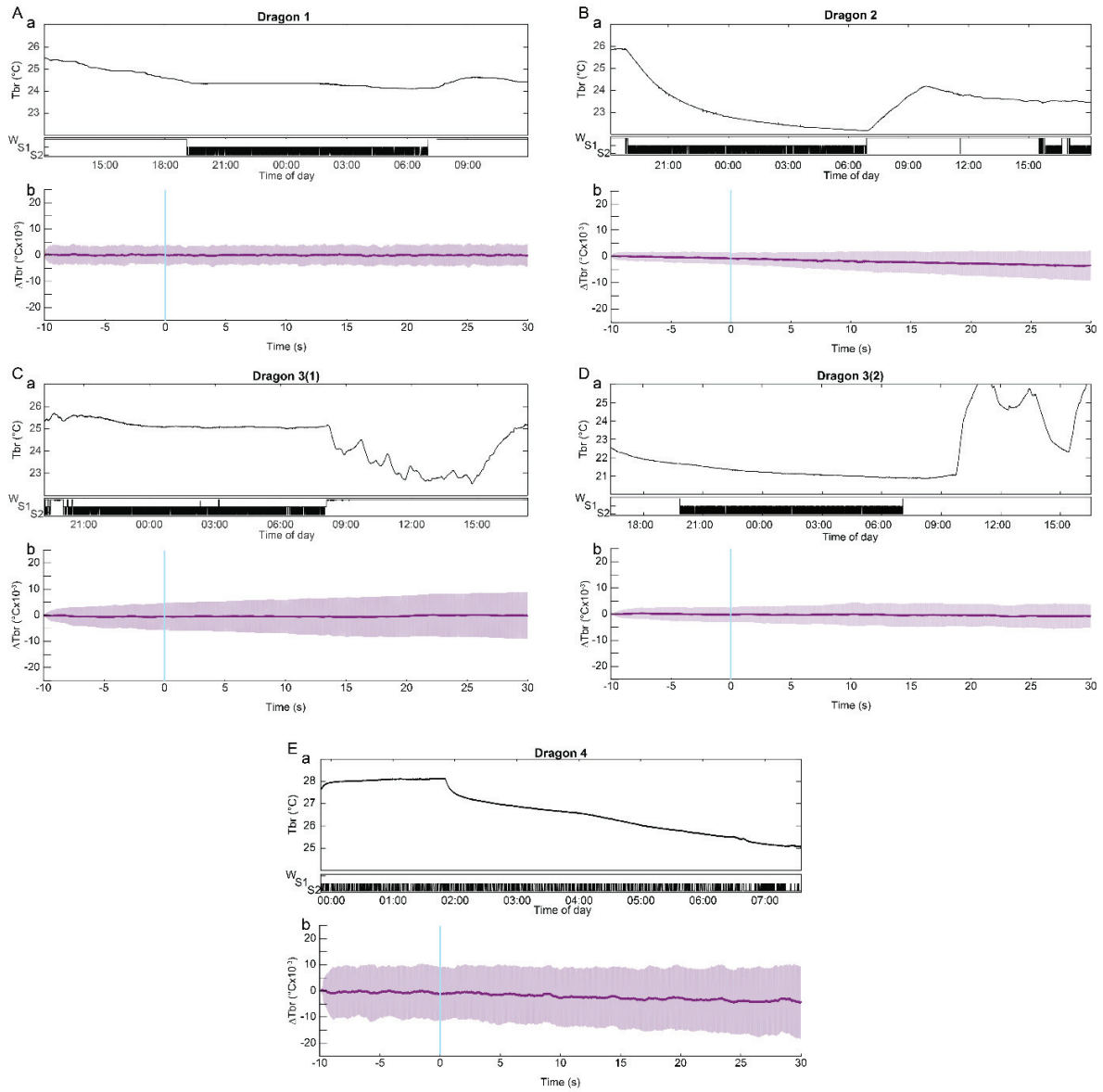
## **Supplemental Information**

### **Comparative Perspectives that Challenge Brain**

### **Warming as the Primary Function of REM Sleep**

**Gianina Ungurean, Baptiste Barrillot, Dolores Martinez-Gonzalez, Paul-Antoine Libourel, and Niels C. Rattenborg**

## Supplemental Information



**Figure S1. Brain temperature ( $T_{br}$ ) across the main night-time sleep period for individual bearded dragons, Related to Figure 3B.** For each individual (A-E; two nights (C and D) are shown for Dragon 3), (a) shows  $T_{br}$  with a hypnogram (bottom) showing brain state (wakefulness, W; NREM-like sleep, S1; REM-like sleep, S2) and (b) shows  $T_{br}$  recorded from the dorsal ventricular ridge 10 s before and 30 s after the transition (vertical blue bar) between NREM-like and REM-like sleep.  $T_{br}$  values (mean  $\pm 1$  standard deviation) are expressed relative to  $T_{br}$  10 s before the transition.



**Table S1. Results of the linear mixed effect models, Related to Figure 1C and D.** For the last four models the predictors were scaled upon fitting the model.

Category	Response	Predictor	Fixed effects			Random effects	
			Estimate $\pm$ SE	t-val	p-val	Proportion (%) of variance explained by:	
						random intercept	random slope
<b>Effect of bout length on <math>\Delta T_{br}</math></b>	$\Delta T_{br}$	<i>Intercept</i>	$-7.30 \cdot 10^{-3} \pm 8.70 \cdot 10^{-4}$	-8.39	$1.56 \cdot 10^{-4}$	12.5	1.68
		NREM bout length	$3.11 \cdot 10^{-3} \pm 3.31 \cdot 10^{-4}$	-9.40	$8.55 \cdot 10^{-5}$		
	$\Delta T_{br}$	<i>Intercept</i>	$7.86 \cdot 10^{-3} \pm 5.70 \cdot 10^{-4}$	13.80	$6.48 \cdot 10^{-6}$	2.80	0.02
		REM bout length	$3.37 \cdot 10^{-4} \pm 5.59 \cdot 10^{-5}$	6.04	$1.04 \cdot 10^{-3}$		
<b>Effect of bout length on the occurrence of min/max <math>\Delta T_{br}</math> relative to bout offset</b>	$\Delta T_{br}$ min occurrence	<i>Intercept</i>	$-0.56 \pm 0.55$	-1.02	0.35	1.56	3.36
		NREM bout length	$-5.93 \pm 0.79$	-7.54	$2.77 \cdot 10^{-4}$		
	$\Delta T_{br}$ max occurrence	<i>Intercept</i>	$9.22 \pm 1.93$	4.79	$3.05 \cdot 10^{-3}$	6.33	0.30
		REM bout length	$3.37 \cdot 10^{-4} \pm 5.59 \cdot 10^{-5}$	-1.98	0.1		
<b>Effect of <math>T_{br}</math> on the REM density</b>	REM density	<i>Intercept</i>	$2.18 \pm 0.18$	12.03	$2.21 \cdot 10^{-5}$	1.85	0.34
		$T_{br}$	$-0.50 \pm 0.08$	-5.99	$1.02 \cdot 10^{-3}$		

## Transparent Methods

### Pigeons

*Surgical procedures:* Adult domestic pigeons (*Columba livia*; Budapest variety; 1 female, 6 males) were surgically instrumented for recording the electroencephalogram (EEG) from both hemispheres, neck electromyogram (EMG), and eye movements under isoflurane anesthesia (1-3% in 0.5 LPM oxygen). The birds were administered an injection of Diazepam (2 mg/kg) into the breast muscle 15 min prior to surgery, an injection of Metamizol (150 mg/kg) into the breast muscle shortly before isoflurane

anesthesia, 4 drops of Procaine (2%) for local analgesia prior to skin incision, and an intramuscular injection of Meloxicam (0.5 mg/kg) and Lidocaine gel to the wound at the end of the surgical procedure. The electrodes consisted of round-tipped gold pins (0.5 mm diameter) for the EEG and electrooculogram (EOG), and gold-plated electrode balls (1 mm diameter) for the EMG. The EEG electrodes were placed over the visual hyperpallium which is visible through a distinct window of thin bone on the surface of the cranium. Two EEG electrodes were placed on the dura overlying each (left and right) hyperpallium (2.5 and 3 mm lateral of the midline for the anterior and posterior electrode, respectively, and separated by 5 to 6 mm along the anterior-posterior axis). As part of another study, left and right pupil size and movements were detected through the closed eyelids via head-mounted micro camera modules (NanEye<sup>®</sup>, ams AG, 1 mm x 1 mm) connected to an image processing board (BAP Image Systems GmbH, AC62KUSB), and an infra-red LED (Kingbright<sup>®</sup>, 940 nm, 50 mA, 1.2 V) placed on the skull that illuminated the back of the eyes (Yüzgeç et al., 2016). For each hemisphere, a thermistor (Semitec<sup>®</sup>, 223F $\mu$ 3122-07U015, 0.28 mm diameter, thermal time constant of 20 ms, relative sensitivity 0.001°C) was inserted approximately 1 mm into the dorsal surface of the left and right hyperpallium, mid-way between the two EEG electrodes. Each thermistor was calibrated prior to implantation using a water bath and a digital thermometer.

*Housing and recording:* The pigeons were housed individually in an indoor aviary (1 m x 2 m x 2 m; 12 h:12 h light:dark photoperiod; 21°C) adjacent to, and in visual and auditory contact with, two other pigeons, each housed in similar aviaries. Food and water were provided *ad libitum*. After at least one week of post-surgical recovery and habituation to the recording equipment, the birds were recorded during the 12-h night while confined to their preferred sleeping perch. Electrophysiological data and head movements (accelerometry) were recorded at 256 Hz via a 2.6 g head-mounted transmitter. Images from the micro cameras were recorded via a thin, light-weight tether that allowed the pigeon to

freely move on its perch. The birds were disconnected from the tether and were free to move throughout their aviary once the lights came on in the morning.

*Sleep scoring:* The recordings were visually scored for wakefulness, NREM sleep, and REM sleep using standard criteria (Martinez-Gonzalez et al., 2008; Lesku et al., 2011; Tisdale et al., 2018). However, unlike previous studies which employed 4 s epochs, we used 1 s epochs to gain a more precise measure of the onset and offset of bouts of REM sleep. A bout of a given state was defined as one or more epochs of that state uninterrupted by an epoch of any other state; i.e. bouts could be as short as 1 s.

*Eye movement tracking:* The videos recordings of the birds' eyes were used to detect the pupil area through binary thresholding using a custom Matlab script. Variations in the position of the pupil area centroid were used as a proxy for eye movements. To correct for variation in camera position, the axes were rotated such that horizontal movements were along and vertical movements were perpendicular to the eyelid closure plane. Positive increases in signal values represent anterior and dorsal movements, respectively.

*Brain temperature analysis:* Although temperature was recorded from both hemispheres, in some birds, state-related changes in temperature were less evident in one hemisphere than the other, even though the changes in EEG activity were largely synchronous between the hemispheres. This variation likely reflects variation in the depth of the thermistor. Consequently, the thermistor that appeared the most responsive to state changes was selected for detailed analysis. Temperature signals were first filtered using a moving mean with a 1.0 s window. Due to frequent state changes and the fact that temperature usually peaked after a bout of REM sleep, we took the following measures to isolate the relationship between sleep state and  $T_{br}$ . For NREM sleep, we analyzed temperature starting 10 s after the onset of a bout. For REM sleep, we selected for analysis the bouts that were preceded and followed by at least 20 s of NREM sleep to avoid the overlapping effect of REM sleep bouts occurring in close succession. For

both states, changes in  $T_{br}$  were expressed relative to the temperature at the start of the analysis window. For Figure 1C, NREM sleep bouts were grouped into 10 s duration categories, and for Figure 1D, REM sleep bouts were grouped into 3 s categories. We only report data for categories that had at least 10 bouts that met the inclusion criteria for every bird. This resulted in seven categories for NREM sleep (10-19, 20-29, 30-39, 40-49, 50-59, 60-69, and 70-79 s) and five categories for REM sleep (1-3, 4-6, 7-9, 10-12, and 13-15 s). All reported values are the mean ( $\pm 1$  standard deviation) of the individual bird means for each category.

*Statistical analyses:* On the non-grouped dataset shown in Figures 1C and D, we detected the timing and the values of the maximum decrease/increase in  $T_{br}$  for each  $T_{br}$  response curve associated with a bout of NREM/REM sleep. In the case of NREM sleep, the detection was limited to 10 s over the maximal length of the respective bout category to filter out potential effects of states occurring after the offset of a NREM sleep bout. We then tested whether longer NREM/REM sleep bouts were associated with higher decreases/increases in  $T_{br}$  using linear mixed effect models with the relative change in  $T_{br}$  ( $\Delta T_{br}$ ) as the response variable and the bout length as predictor. Individual identity was added as a random effect. A similar analysis was performed to test for a relationship between bout duration and the timing of the positive peaks relative to the offset of REM sleep bouts.

If one function of REM sleep is to warm the brain, then REM sleep should be expressed in higher proportions at lower  $T_{br}$ . We therefore tested for a relationship between  $T_{br}$  and the density of REM sleep during 15 s windows. For each bird, we divided the recording into 15 s windows. We then calculated the amount of REM sleep expressed in each 15 s window, and the average  $T_{br}$  for the first 7 s of the associated  $T_{br}$  data. The average for  $T_{br}$  was restricted to the first 7 s to account for the lag in response related to REM sleep bouts. The relationship was tested using a linear mixed effect model with REM sleep density as the response variable and  $T_{br}$  as predictor. Individual identity was added as a random effect. All statistical analyses were performed using RStudio. For mixed model analyses, we used

the lme4 package (Bates et al., 2015). The denominator degrees of freedom were computed with Satterthwaite's method.

### Bearded dragons

*Surgical procedures:* 4 adult bearded dragons (*Pogona vitticeps*, 2 males, 2 females) were surgically instrumented for recording local field potentials (LFP) in the DVR, neck EMG, and eye movements under injectable ketamine (100 mg/kg) and medetomidine (200 µg/kg) anesthesia. The LFP were recorded using a bundle of six 35-µm diameter tungsten electrodes. The EMG was recorded using two stainless steel electrodes implanted in the neck muscles. The eye movements were recorded using one screw fixed in the skull on the supraorbital ridge of each eye. A calibrated thermistor (Semitec®, 223Fµ3122-07U015; or Micro-Betachip, GA100K6MCD1; the sensitivity of both sensors was around 0.001°C with a time constant of 20 ms) was inserted in the telencephalon 1 mm into the brain, targeting the DVR. The thermistor and LFP electrodes were positioned relative to the pineal eye (anterior-posterior (AP) 0 mm, medial-lateral (ML) 0 mm) and the surface of the skull (dorsal-ventral (DV) 0 mm). The thermistor positions were as follows: Dragon 1, AP -1 mm, ML 0 mm, DV -2.5 mm; Dragon 2, AP +1 mm, ML -1 mm, DV -2.5 mm; Dragon 3, AP -2 mm, ML -2 mm, DV -2.5 mm; and Dragon 4, AP +1 mm, ML +1mm, DV -2.5 mm. The LFP electrodes were placed bilaterally (AP -2 mm, ML +2 mm and -2 mm, and DV -5 mm).

*Housing and recording:* The lizards were housed individually in a glass terrarium (50 cm x 50 cm x 100 cm; 12 h:12 h light:dark photoperiod). Water was provided *ad libitum* and food (crickets, mealworms, and vegetables) was given every other day. After at least one week of post-surgical recovery and habituation to the recording equipment, the lizards were recorded for 3 to 7 d in their terrarium. Electrophysiological data and head movements (accelerometry) were recorded at 256 Hz via a 15g head-mounted transmitter (Massot et al., 2019). The animals were recorded at different room temperatures. Dragon 1 was recorded at a constant temperature (25°C), while the others were maintained with a more

natural temperature gradient between a 'hot spot' (set at 40°C) and a 'cold spot' (25°C). Lizards housed with a gradient exhibited a natural decrease of their core temperature across the sleep period (Figure S1).

*Sleep scoring:* The recordings were visually scored for wakefulness and sleep using body position, head elevation, and eye state (open or closed). The animals were considered asleep when in a "sleeping" position (lying on the ground with hind and front limbs relaxed) with the head on the ground and both eyes closed for more than a minute. The two sleep states were scored using a method previously developed for lizards (Libourel et al., 2018).

*Brain temperature analysis:* Temperature signals were first filtered using a moving mean with a 0.5 s window. To estimate the impact of sleep state on  $T_{br}$ , we examined  $T_{br}$  10 s before the onset of REM-like sleep to 30 s after onset. All episodes of REM-like sleep occurring across the night were included in the analysis. The values plotted in Figure 3B are the mean of the nightly means (N = 5; one night for Dragons 1, 2, and 4, and two nights for Dragon 3); for individual bearded dragon data, see Figure S1.

#### Ethics statement

All experiments were conducted according to the 3R principles of animal experimentation and in accordance with the European Community Council Directive for the use of research animals. The protocols and procedures used were approved by the local ethics committee for animal experimentation (i.e., Government of Upper Bavaria for the pigeons and University Lyon 1 for the bearded dragons).

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Supplementary II – Intramuscular Administration of Ketamine-Medetomidine  
Assures Stable Anaesthesia Needed for Long-Term Surgery in the Argentine  
Tegu, *Salvator Merianae*

Baptiste Barrillot, Johanna Roux, Sébastien Arthaud, Laëticia Averty, Angeline Clair, Anthony  
Herrel, Paul-Antoine Libourel

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Author(s): Baptiste Barrillot, M.Sc., Johanna Roux, M.Sc., Sébastien Arthaud, B.Sc, Laëtitia Averty, B.Sc., Angeline Clair, B.Sc., Anthony Herrel, Ph.D., and Paul-Antoine Libourel, M.Sc.

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## INTRAMUSCULAR ADMINISTRATION OF KETAMINE-MEDETOMIDINE ASSURES STABLE ANAESTHESIA NEEDED FOR LONG-TERM SURGERY IN THE ARGENTINE TEGU *SALVATOR MERIANAE*

Baptiste Barrillot, M.Sc., Johanna Roux, M.Sc., Sébastien Arthaud, B.Sc., Laëtitia Averty, B.Sc., Angeline Clair, B.Sc., Anthony Herrel, Ph.D., and Paul-Antoine Libourel, M.Sc.

**Abstract:** To define a protocol of anesthesia for long-duration invasive surgery in a lizard, eight young adult Argentine tegus (*Salvator merianae*) of mean body weight 3.0 kg (interquartile range [IQR] 3.40–2.65) were anesthetized with a mixture of ketamine (K) and medetomidine (M) at 19°C, injected intramuscularly and equally distributed in the four limbs. As the experimental surgery procedure required a prolonged deep anesthesia with a good myorelaxation (between 16 and 21 hr), reinjections were required and reflexes were checked during surgery. Times for anesthetic induction, anesthetic reinjection, and recovery periods were recorded for five different combinations of ketamine-medetomidine: 1) 66 mg/kg K + 100 µg/kg M; 2) 80 mg/kg K + 100 µg/kg M; 3) 100 mg/kg K + 130 µg/kg M; 4) 125 mg/kg K + 200 µg/kg M; and 5) 150 mg/kg K + 200 µg/kg M. The effect on the recovery speed of the postoperative atipamezole injection was also evaluated. The median induction time was 30 (IQR 35–27.5) min with no statistical difference between all the concentrations tested. The first reinjection of half a dose was administered after a mean of 5 hr (5.64 hr, IQR 5.95–4.84) as were the subsequent reinjections of a quarter dose (3.99 hr, IQR 5.98–3.23). Intramuscular administration of the ketamine-medetomidine combination is a simple, rapid, and efficient anesthesia for long-term surgery (>12 hr). A mix of 100 mg/kg ketamine and 200 µg/kg medetomidine, with reinjections every 4 hr of half a dose of the previous injection can maintain a good quality of anesthesia for at least 16 hr. The injection of atipamezole after the surgery reverses the effects of medetomidine and permits a reduction of the recovery period.

**Key words:** Anesthesia, ketamine, medetomidine, reptile, *Salvator merianae*.

### INTRODUCTION

The number of exotic pets encountered in veterinary clinics is constantly growing.<sup>4</sup> This increase raises the problem of the care for these species, especially when a major surgical intervention is needed. In those cases, inducing a prolonged and stable anesthesia of the animal is necessary. Unfortunately, no published protocol on how to induce and maintain prolonged deep anesthesia in lizards appears to exist. The use of

inhalant anesthetic agents, such as isoflurane or halothane, is common in lizards for small interventions but it requires expensive and dedicated equipment to ensure the best anesthesia.<sup>15</sup> Moreover, when prolonged anesthesia is required these methods tend to desiccate the airways if the anesthetic is not hydrated. Furthermore, intubation renders surgery to the head or buccal cavity difficult and inhalant anesthesia cannot be easily used in the wild. Injectable anesthetics are a good and straightforward alternative but their use and dosage for prolonged anesthesia has not been described in the literature.<sup>3</sup> The focus of the present study was on establishing an injection protocol using a mix of ketamine and medetomidine for the anesthesia of a species of large squamate, *Salvator (Tupinambis) merianae*.<sup>10</sup>

Although many injection protocols have been proposed, dissociative anesthetics appear to be the most appropriate because these agents induce a state of catalepsy, a sort of paralysis in which the animal is dissociated from its environment.<sup>2</sup> Historically, ketamine has been one of the most commonly used injectable dissociative agents by veterinarians for “reptile” anesthesia due to its effective induction time and some degree of

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From the Neuroscience Research Center of Lyon, CNRS UMR 5292, INSERM U1028, Team SLEEP, Faculté de Médecine Laennec, 7, Rue Guillaume Paradin, 69372 Lyon, France (Arthaud, Barrillot, Libourel, Roux); Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés, CNRS, UMR 5023-LEHNA, 6, Rue Raphaël Dubois-Bât. Forel, 69622 Villeurbanne, France, (Averty, Clair); Département Adaptations du Vivant, UMR 7179 CNRS/MNHN, 57 Rue Cuvier, Code Postale 55, 75231 Paris Cedex 05, France (Herrel); Evolutionary Morphology of Vertebrates, Ghent University, K.L. Ledeganckstraat 35, B-9000, Gent, Belgium, (Herrel). The authors recognize that Barrillot and Roux contributed equally to this work. Correspondence should be directed to Libourel (pa.libourel@univ-lyon1.fr).

immobilization.<sup>18</sup> Ketamine is a noncompetitive antagonist of the NMDA receptor, which plays an important role in the modulation of neurotransmission and neuron excitability.<sup>22</sup> This characteristic may explain its dissociative effect because it affects the activity of the limbic system. Nonetheless, this product requires a high dose (170–230 mg/kg), involves a long recovery time,<sup>1</sup> and can induce many side effects when used as a sole agent (increase in muscle tone, hypersalivation, acceleration of apoptosis when administered in high doses).<sup>6,13</sup> Consequently, an  $\alpha_2$  adrenergic agonist, such as medetomidine, is often used in combination with ketamine. Indeed, medetomidine has an analgesic effect and myorelaxant properties, and increases the total anesthesia period.<sup>9</sup> The sedative effect of medetomidine is due to the particular action of this  $\alpha_2$ -agonist on the norepinephrine release in the central nervous system. The norepinephrine liberation is blocked by medetomidine and therefore induces a profound sedation. Moreover,  $\alpha_2$ -agonists also act on the spinal cord by inhibiting the interneurons causing a significant myorelaxation. Additionally, their effects on the brain and spinal cord play a key role in inhibiting the pain pathways and inducing analgesia.<sup>20</sup> Furthermore, medetomidine can be reversed with atipamezole, an adrenoceptor antagonist.<sup>17</sup> Another advantage of these products is that they can be mixed together and injected intramuscularly (im).<sup>7</sup> The intramuscular route is the most commonly used injection route for “reptiles” because it is also the most practical one. Moreover, the im injection avoids the high peak concentration common in iv injection and prevents the harmful side effects this peak could induce.<sup>8</sup> The use of medetomidine furthermore permits a decrease in the dose of ketamine and an optimization of the recovery time.<sup>18</sup> Thus, it is possible to immobilize “reptiles” for a long duration, as when needed in the context of a major surgery.

The hypothesis is that a mix of ketamine and medetomidine could be used to induce a prolonged and stable anesthesia in lizards for a long-term intervention. Therefore different concentrations were tested to determine the optimal protocol.

## MATERIALS AND METHODS

All experiments were conducted applying the 3R principles in animal experiments and in accordance with the European Community Council Directive for the use of research animals (86/609/EEC and 2016/63/EU; <http://ec.europa.eu/environment/>

[chemicals/lab\\_animals/pdf/endorsed\\_awb-nc.pdf](#)). Protocols and procedures used were approved by the local Comité d’Ethique en Experimentation Animale of the University Lyon 1.

### Animals and surgery

Eight adult (1.75 yr, interquartile range [IQR] 2–1.5; two females) Argentine tegus, *Salvator merianae*, (3.0 kg, IQR 3.40–2.65) were used for the experiments. In order to record different physiological parameters simultaneously, a prolonged deep anesthesia with a good myorelaxation was needed. The surgery involved clearing off the top of the skull and multiple trepanations, small skin and muscles incisions, as well as the placement of deep brain electrodes.

### Monitoring

To assess the depth of the anesthesia, several reflexes were checked regularly. The absence of the spinal reflexes (eg failure to breathe when a finger is run down the back and failure to move the tail when pinched) and the palpebral reflex (ie closing of the eyelid when touched) were considered as being signs of a good anesthesia. The conservation of the corneal reflex was also used to ensure the right depth of the anesthesia, as well as the breathing rate, which was evaluated regularly and visually during the surgery. During the recovery, all animals were kept under observation using infrared cameras 24 hr a day.

### Surgery conditions and anesthetics

All surgeries started in the morning, between 6:00 and 8:00 AM, in a temperature-controlled room ( $19 \pm 2^\circ\text{C}$ ). The first injection and recovery took place in the home enclosure of the animal in order to reduce stress (room temperature  $25^\circ\text{C}$ ). The hot spot at  $45^\circ\text{C}$  was turned off 1 day before the surgery and turn on again 3 days after.

The anesthetics used for the experiments were ketamine (Kétamine 1000 Virbac; Virbac, 06517 Carros, France) at five different dosages (66, 80, 100, 125, and 150 mg/kg), and medetomidine (Domitor, Vétoquinol; Orion, 02200 Espoo, Finland) at three different dosages (100, 130, and 200  $\mu\text{g}/\text{kg}$ ). Ketamine concentrations were based on prior experiences with ketamine as a sole induction agent during surgery in different lizards and experience with short-duration surgery using both ketamine and medetomidine.<sup>5,11,12,14</sup> The extemporaneous mixture was injected im in the triceps of the anterior limbs and the iliotibialis muscle of the posterior limbs.

**Table 1.** Anesthesia protocol per animal. Summary of the anesthesia, the weight of the animal, and anesthetic products and concentrations used for the surgery. The induction time of the anesthesia is also provided for each animal.

Animal	Weight (kg)	Ketamine (mg/kg)	Medetomidine ( $\mu\text{g}/\text{kg}$ )	Induction time (min)
Animals 1 to 4	2.8 $\pm$ 0.8	66	100	24.8 $\pm$ 11
Animal 5	3	80	100	30
Animal 6	3	100	130	58
Animal 7	3.75	125	200	35
Animal 8	2.8	150	200	30

The median injected volume during the surgery was 2.75 ml (IQR 3.79–2.31) and was equally distributed in the four limbs of the animal to prevent pain. Two groups of dosages were defined: a high-dose group with a ketamine dosage between 100 and 150 mg/kg and a low-dose group with a ketamine dosage ranging from 66 to 80 mg/kg. Reinjection of anesthetics were decided when any signs of awakening were observed (eg increase in respiratory rate). Each reinjection was half the concentration of the previous one. In some cases ( $n = 4$ ), atipamezole (Antisedan, Vétoquinol; Orion, 02200 Espoo, Finland) was injected im in the iliotibialis muscle of the posterior limbs, to reverse the effect of medetomidine, the concentration being once or twice the concentration of medetomidine (ie 100–400  $\mu\text{g}/\text{kg}$ ). It was injected 12 hr after the surgery.

#### Data analysis

All statistical analyses were performed using MATLAB® (MathWorks®, Natick, Massachusetts 01760, USA). The difference in the induction time and the reinjection time between the two dosage groups was analyzed using Mann-Whitney  $U$ -tests. Differences were considered significant at  $P < 0.05$ . All data are expressed as median (IQR third quartile to first quartile).

## RESULTS

### Induction of anesthesia

Anesthetic concentrations and induction times are presented in Table 1. No statistical difference in induction time between low and high concentrations was observed ( $P = 0.14$ ). The median induction time was about 30.0 (IQR 35.0–27.5) min.

### Reinjection

For the eight lizards, there were about 2.00 (IQR 2.25–1.75) reinjections needed during the

surgery. The first reinjection of anesthetic was around 5.64 (IQR 5.95–4.84) hr, and in the majority of cases, half a dose was reinjected. The median time between the following reinjections was 3.99 (IQR 5.98–3.23) hr with a concentration of half of the previous injection. There was no statistical difference for the time of reinjection between low and high doses of anesthetic ( $P = 0.88$ ). The results are summarized in Figure 1. The surgeries lasted 15.8 hr (IQR 17.0–14.7) without any signs of awakening for all animals.

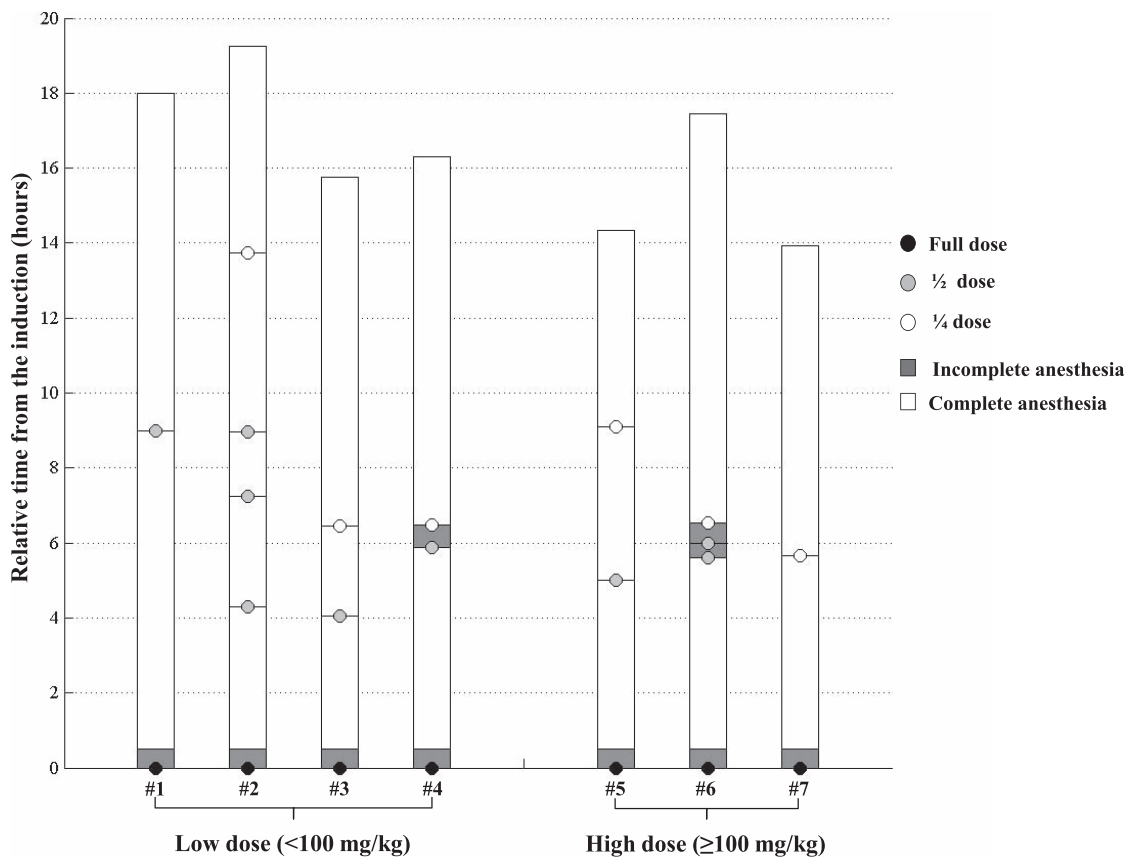
### Recovery time

Spinal and palpebral reflexes started to appear a few hours after the last injection of anesthetic. Twelve hours after the end of the intervention, signs of consciousness including eye opening, limb movements, and tongue flicks were present. Animals that received atipamezole at this time became more reactive to stimulation. However, all animals preferred to rest with a full voluntary locomotion appearing 3 days (IQR 3.50–3.00) after the last anesthetic dose. After surgery all animals displayed normal behavior including, feeding, locomotion, and sleeping, and were monitored for up to 3 mo consecutively. No signs of pain (eg prostration, increase in aggressiveness, stereotypic movements), stress, or ill health were observed after surgery.<sup>16</sup>

## DISCUSSION

The aim of the study was to determine an anesthesia protocol allowing maintenance of a prolonged anesthesia needed to perform an invasive surgery on a large lizard, *Salvator merianae*. To do so, different concentrations of a mix of ketamine and medetomidine were tested, as those anesthetics allow a deep and stable anesthesia.<sup>18</sup>

The results showed that the protocols induce a rapid and deep anesthesia (median induction time 30 min, IQR 35.0–27.5) and that the induction time was not significantly modified by the dosage



**Figure 1.** Timing of the anesthesia. Relative time (hr) from the first injection for each surgery ( $n = 7$ ). The black, grey, and white markers indicate the concentration and time of an injection or reinjection. White rectangles represent a state of good and deep anesthesia whereas the grey ones represent a state of incomplete anesthesia (induction or animal not completely unconscious or myorelaxed).

of the anesthetic (Table 1). The depth of the anesthesia was regularly controlled by measuring the breathing rate and the assessment of different reflexes (palpebral, spinal, corneal). The protocol induced a deep anesthesia that was maintained for 5 hr (5.64 hr, IQR 5.95–4.84; Fig. 1).<sup>2</sup> If the half-dose reinjection is given too late during the anesthesia plateau, the time necessary to reinduce a proper anesthesia can be considerably increased, however. Therefore, it is crucial to not exceed 5 hr before the first reinjection. If the lizard presents any signs of arousal, a half dose is required; otherwise, a quarter dose suffices. Complex surgeries require that the animal stays unconscious for a long period of time and the protocol described here grants this. It is worth mentioning that, unlike what could be found in the literature, the protocol uses higher ketamine doses (i.e., >60 mg/kg) as these are needed to induce a deep anesthesia, essential for a stereo-

tactic surgery, for example.<sup>2</sup> The robust dissociative effect of those concentrations allows for an invasive procedure without the risk of a subject awaking midsurgery.

Secondly, the im injection of atipamezole postoperatively tended to reduce the time to the reappearance of consciousness. The injection 12 hr after the intervention permitted maintenance of relative immobility of the animal, by keeping the myorelaxant effect of the medetomidine. However, with or without atipamezole, the animal recovered all its spinal and palpebral reflexes within 12 hr after the last injection. Interestingly, animals preferred resting for several days (3 days, IQR 3.5–3). Personal experience with single injections of ketamine and medetomidine without surgery (eg during magnetic resonance imaging), followed by an injection of atipamezol a few hours after, was associated with a recovery time (exploration behavior, voluntary locomotion, feeding,

and tongue flicking) that was much shorter, with a complete recovery within the next 12 hr. In this protocol the atipamezol was injected in the middle of the night. Consequently, the rest induced by the anesthesia cannot be differentiated from the rest associated with sleep. However, as preliminary experiences with smaller species suggest a recovery time within the hour after an injection of atipamezol, a species-specific reaction of the tegu cannot be excluded. The long recovery period could be a limitation when needing to use anesthesia in the wild and further investigation is needed to properly assess this issue. Furthermore, the reversal potential of atipamezole is dose-dependent and it could be interesting to test the effect of a higher dosage of this product on the recovery time.<sup>19</sup>

This anesthesia protocol allowed performance of complex and major surgery on a lizard (*Salvator merianae*) under stable anesthesia. All animals ( $n = 8$ ) recovered well after the surgery. As the metabolism of tegus and other poikilotherms is temperature dependent, and as a consequence also the pharmacokinetic effect, particular attention should be paid to the room temperature.<sup>21</sup> For a long surgery, the anesthesia should be done at a relatively low temperature (around 20°C) without any heating device, in order to reduce the degradation of the anesthetic, thus extending the anesthesia. Moreover, an increase of the recovery room temperature could decrease the recovery time. Therefore, when used in the wild, the anesthesia and recovery period will likely be affected by the ambient temperature.

### CONCLUSIONS

This study suggests that the use of a mixture of 100 mg/kg of ketamine combined with 200 µg/kg of medetomidine injected im is adapted to inducing a deep anesthesia in the tegu. At 20°C this protocol provides immobility and a stable anesthesia for 5 hr. After that, reinjections of half the previous dose every 5 hr are recommended if required. The injection of atipamezole (400 µg/kg) after surgery contributes a reduction of the recovery period. The good response to the anesthetics and the satisfactory recovery of the animal suggests that this protocol induces a sufficiently prolonged and deep anesthesia in the Argentine tegu and could be transposed to other species of lizards.

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Figure S1 – Mean HvW wave form per vigilant state of the *Pogona vitticeps*. Each row is a different 24h recording. Row 1 to 3 are of Animal 1. Row 4 to 7 are of Animal 2. Row 8 & 9 are of Animal 3. Blue = Active Wake, Orange = Quiet Wake, Green = S1, Red = S2.



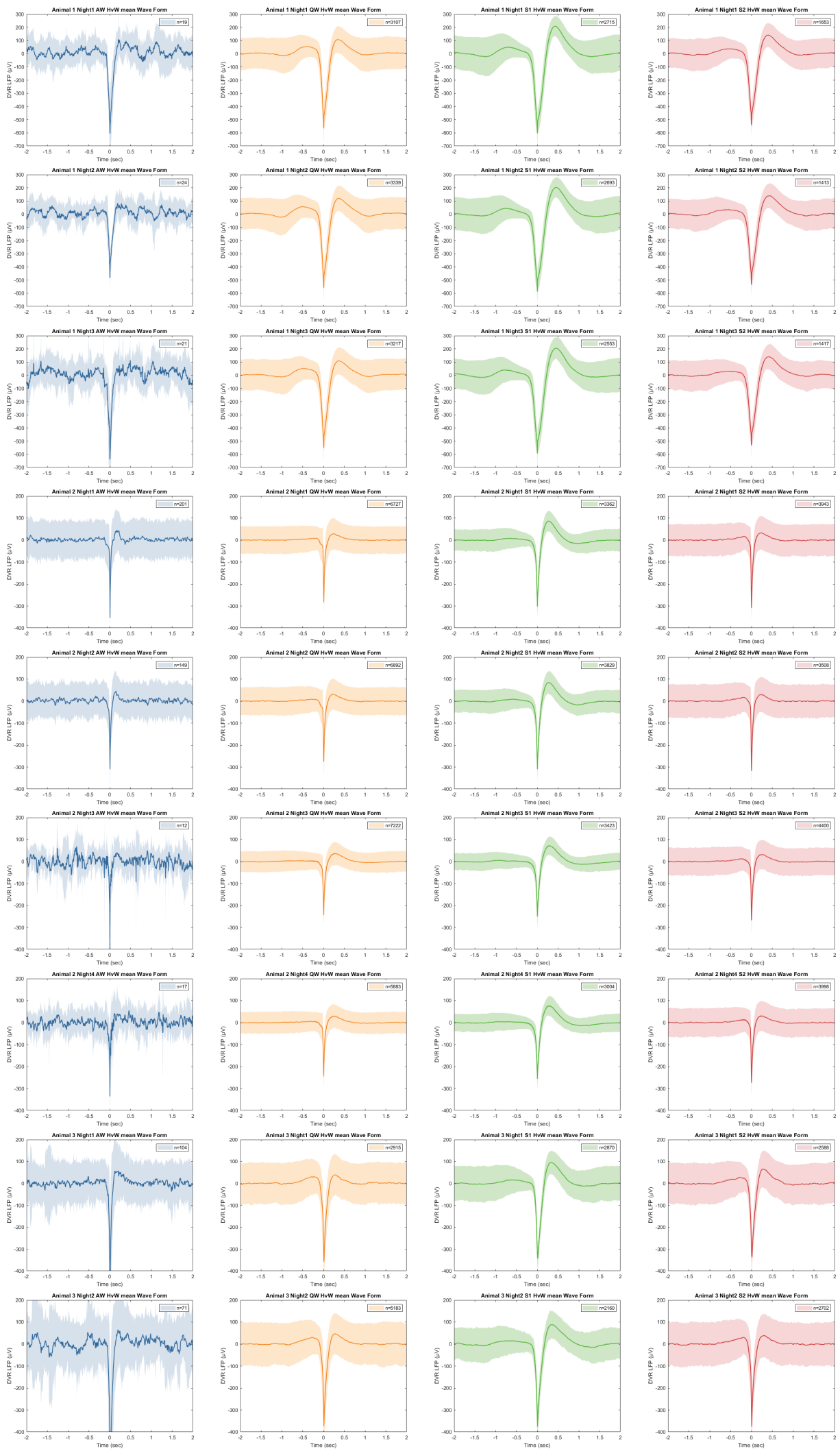




Figure S2 – Mean HvW wave form per vigilant state of the *Stellagama stellio*. Each row is a different 24h recording. Row 1 to 4 are of Animal 1. Row 5 to 7 are of Animal 2. Blue = Active Wake, Orange = Quiet Wake, Green = S1, Red = S2.

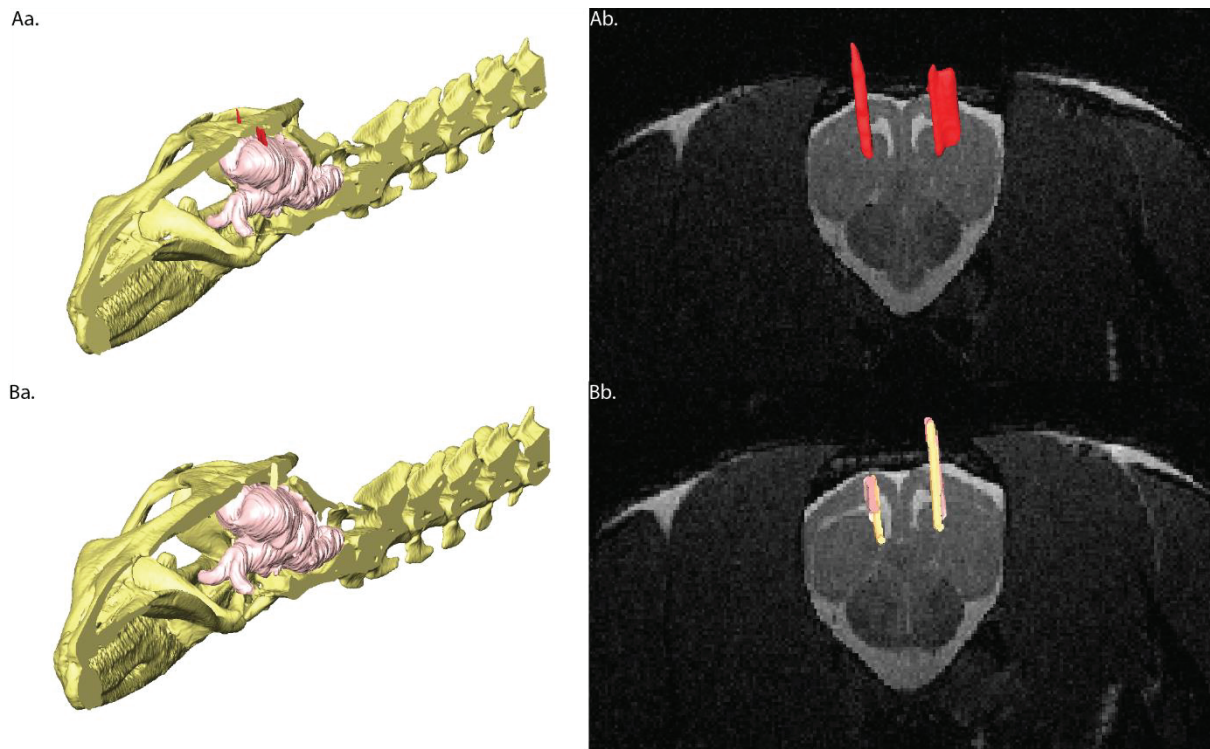
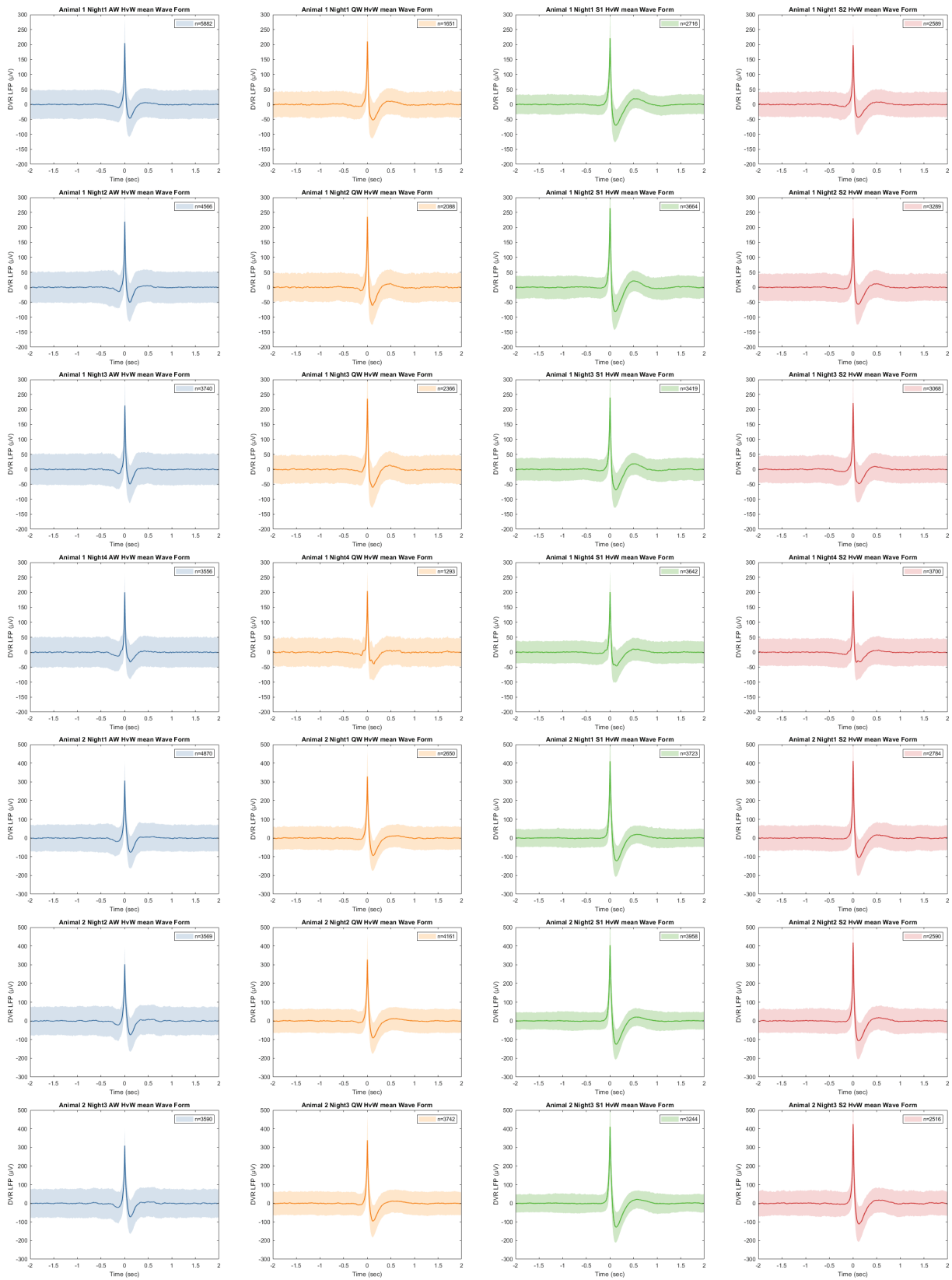
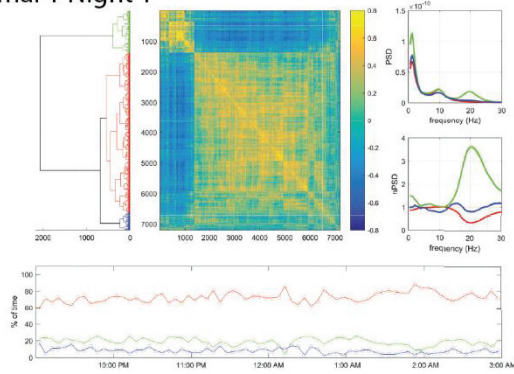


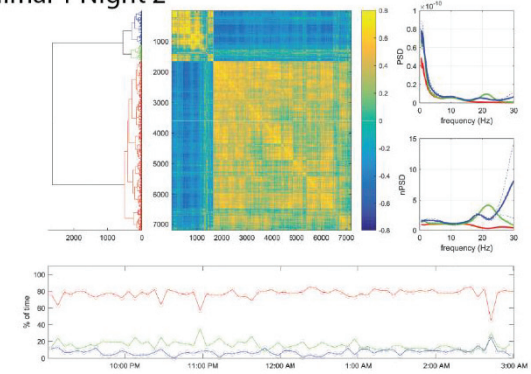
Figure S3 – Anatomical MRI and CT scan reconstructions of the brain and skulls of our two *Stellagama stellio* after implantation (A. Animal 1, B. Animal2). For each individual, a. 3D rendering of the skull (light yellow), brain (light pink), and LFP bundles (Aa. Red, Ba. Light beige) b. MRI coronal section of the DVR with the reconstructed LFP bundles positions (Ab. Red, Bb. Beige and pink).



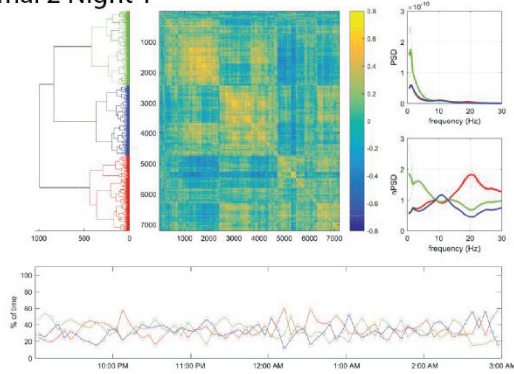
A. Animal 1 Night 1



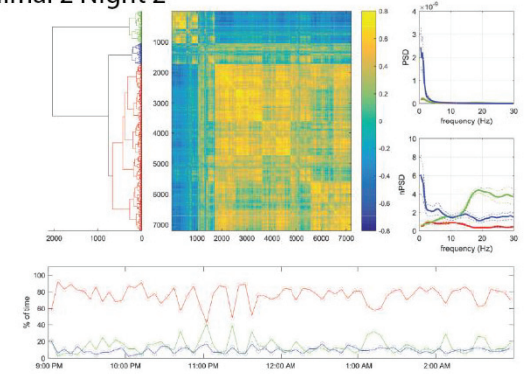
Animal 1 Night 2



Animal 2 Night 1



Animal 2 Night 2



B.

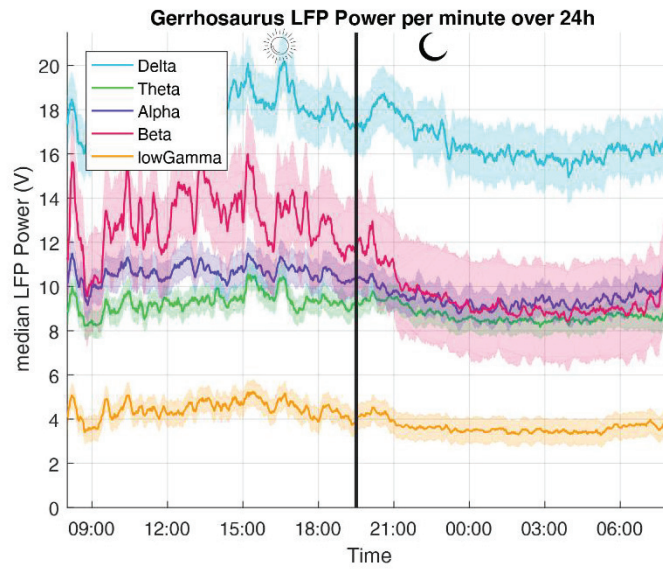


Figure S4 – A. Clustering analysis for three brain oscillations of two of the Broadleysaurus major for two different night. With the unsupervised hierarchical clustering, the power spectrum of the three electrophysiological states between 0 & 30 Hz, the normalised PS of the three states, and the relative quantities of each state per 5 min over 6 hours (21:00 to 03:00). B. Median evolution of all frequency bands over 24h in the Broadleysaurus major.





